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Otimização da produção de D(-) ácido lático por *Sporolactobacillus nakayamae e Lactobacillus delbrueckii* utilizando subprodutos agro-industriais

SUSAN MICHELZ BEITEL

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

Rio Claro

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TÍTULO DA TESE: **OTIMIZAÇÃO DA PRODUÇÃO DE D(-) ÁCIDO LÁTICO POR *Sporolactobacillus nakayamae* e *Lactobacillus delbrueckii* utilizando subprodutos agro-industriais**

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Dedicatória

Ao meu querido pai Jaime Beitel,

dedico.

“Faça o que você pode, com o que você tem, no lugar onde você está”

Theodore Roosevelt

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RESUMO

O ácido lático tem sido alvo de ampla investigação devido a sua ampla possibilidade de aplicações, despertando interesse em vários setores biotecnológicos e industriais. Pode ser obtido através de via química ou fermentativa; a vantagem da produção por fermentação é a obtenção de isômeros opticamente puros. Um dos principais interesses é reduzir os custos de produção, assim como desenvolver processos que não gerem resíduos. Os substratos utilizados na fermentação e a etapa de purificação do ácido lático são os principais responsáveis pelo custo do processo. Para tanto, o objetivo do atual estudo foi selecionar substratos disponibilizados por baixo custo, assim como estudar as melhores condições de fermentação que propiciem uma eficiente produção de D(-) ácido lático por cepas selvagens de *Sporolactobacillus nakayamae* e *Lactobacillus delbrueckii*. Os componentes dos meios de cultivo, assim como suas respectivas concentrações foram selecionados a partir de planejamentos experimentais. Por sua vez, foi avaliada a influência de diferentes agentes neutralizadores de pH na fermentação. Também foram estudados os parâmetros fermentativos como pH, temperatura, agitações e proporções de inóculo. A fim de elevar os níveis de produção e produtividade, foram estudadas diferentes estratégias de bateladas alimentadas. Foi desenvolvido um processo de purificação utilizando recursos de baixo custo. A partir do meio de cultivo composto de açúcar cristal, farinha de amendoim e Tween 80, *S. nakayamae* foi capaz de produzir 112,93 g/L de D(-) ácido lático. A purificação e recuperação do ácido lático foram realizadas por filtração do meio fermentado em carvão e celite, em seguida passado por resina de troca catiônica. Essa técnica revelou-se eficiente na remoção de açúcares e proteínas assim como na recuperação do ácido lático. Ainda utilizando *S. nakayamae* outro meio de cultivo foi estudado, a partir da mesma fonte de carbono, porém usando extrato de levedura (subproduto da produção de etanol) como fonte de nitrogênio e adicionado de alguns sais. Após estudar as melhores condições de cultivo em biorreator (pH 6.0, 35 ºC, 20% de volume de inóculo e 125 rpm), alcançou-se uma produção de 122,41 g/L de ácido lático e produtividade de 3,65 g/L.h por fermentação em batelada alimentada multi pulsos, em 54 horas. O agente controlador de pH selecionado foi NaOH, que colabora evitando a geração de resíduos durante a purificação. A produção de D(-) ácido lático foi estudada por um segundo micro-organismo (*L. delbrueckii*), desenvolvendo-se um processo fermentativo eficiente, de baixo custo, a partir do qual os mais altos níveis de produção (162 g/L) foram alcançados. O meio de fermentação foi composto de melaço de cana-de açúcar, água de maceração de milho e alguns sais, tais resíduos foram utilizados sem necessidade de

hidrólise. A fermentação foi conduzida por 48 horas em batelada alimentada multi pulsos a 39 ºC, 150 rpm, 25% de inóculo, pH 5.5, controlado por $Ca(OH)_2$ que, além de ser economicamente viável, apresenta a vantagem de não aumentar drasticamente a pressão osmótica do meio de cultivo. A produção de D(-) ácido lático de elevada pureza óptica a partir dos resíduos agro-industriais utilizados, é uma alternativa econômica para o processo em escala industrial.

Palavras-chave: D(-) ácido lático. Delineamento experimental. Fermentação. Batelada alimentada. *Sporolactobacillus nakayamae*. *Lactobacillus delbrueckii*.

ABSTRACT

Lactic acid has been extensive studied due its wide range of applications, receiving more attention in several industrial and biotechnological fields. This molecule can be obtained by chemical or fermentative pathways; only by fermentation the optically pure isomers could be achieved. Reduce the fermentation cost as well residues generate is a real need. The substrates used in fermentation and the purification step are the mainly responsible for the costs of the process. Therefore, the aims of this study were selected affordable substrates and study the fermentation conditions to improve D(-) lactic acid production by wild strains of *Lactobacillus delbrueckii* and *Sporolactobacillus nakayamae*. In order to evaluate the components media and its ideal concentration, experimental designs were applied. The influence of temperature, pH, inoculum percentage, agitation conditions and pH controlling agents were also verified. Moreover fed-batch feeding strategies were studied in order to increase production and productivity levels of D(-) lactic acid. A purification technic based in low-cost material was developed. D(-) lactic acid concentration of 112.93 g/L was obtained using commercial sucrose, peanut flour, and Tween 80 by *S. nakayamae*. The purification and recovery of D(-) lactic acid from this fermentation medium were performed using filtration on activated carbon, followed by celite and cation resin exchange. This approach showed a high efficiency in protein and sugar removal as well in D(-) lactic acid recovery from the fermentation medium. Other culture media was developed using *S. nakayamae*, achieving 122.41 g/L of D(-) lactic acid and 3.65 g/L.h of productivity by multi-pulse fed-batch fermentation at 54 h, using initial media containing yeast extract (byproduct of alcohol production), crystallized sugar and salts. The best parameters fermentation setting to this process were pH 6.0, 35 °C, 20% inoculum and 125 rpm. The pH agent controller selected was NaOH, which is interesting to avoid future waste generation on purification step. The production from other microorganism (*L.delbrueckii*) was studied, describing an efficient and very low cost process, using molasses and corn step liquor as substrate. A maximum D(-) lactic acid concentration of 162 g/liter was achieved at 48 hours of fermentation, the productivity at this point is 3.37 g/L.h. It was found that the optimal conditions to run this process were 39 ºC, 150 rpm, 25% size of inoculum (v/v) and pH 5.5 controlled by addition of $Ca(OH)_{2,}$ which provides economy to process and prevent cell stress that could be caused by high osmotic pressure of the culture media. The production of D (-) lactic acid of high

optical purity from agro-industrial wastes is an economical alternative process at industrial scale.

Keywords: D(-) lactic acid. Experimental design. Fermentation. Fed-batch fermentation. *Sporolactobacillus nakayamae*. *Lactobacillus delbrueckii*.

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Anexo

Lista de abreviaturas e siglas

%	Por cento
ANOVA	Análise de variância
ATP	Adenosina Trifosfato
D(-)	Levógiro
D-LDH	D-lactato Desidrogenase
FFD	Fractional Factorial Designs
g	Grama
GYP	Glucose, Yeast and Peptone
h	hora
HPLC	High Performance Liquid Chromatography
L	Litro
L(+)	Dextrógiro
LDH	Lactato Desidrogenase
L-LDH	L-lactato Desidrogenase
LR	Lactato Racemase
mg	miligrama
mL	Mililitros
ºC	Graus Celsius
PBED	Plackett-Burman Experimental Design
PDLA	Poli D- ácido lático
PDLLA	Poli DL- ácido lático
pH	Potencial hidrogeniônico
PLA	Polímero de Ácido Lático
PLLA	Poli L-ácido lático
rpm	rotação por minuto
RSM	Response Surface Methodology
V	volume
$Y_{p/s}$	Rendimento produto substrato

Sumário

1 Introdução

O ácido lático é uma das moléculas mais investigadas nos últimos tempos apresentando diversas aplicações na área médica, alimentícia, farmacêutica, ortodôntica, têxtil, entre outras (WEE et al., 2004). Grande atenção tem sido voltada para a produção do biopolímero de ácido lático (PLA). Diferentes polímeros podem ser construídos, apresentando características distintas direcionadas para determinadas aplicações. Isso é possível pelo fato de existirem dois isômeros da molécula de ácido lático, D(-) e L(+) (GHAFFAR et al., 2014). A proporção de cada isômero na constituição dos polímeros é responsável pela característica que o mesmo apresentará, polímeros resistentes ou maleáveis, duráveis ou biodegradáveis podem ser elaborados. O ácido lático pode ser obtido através de via química ou fermentativa, a vantagem da fermentação por micro-organismos é a obtenção de um produto opticamente puro, alcançado pela seleção de cepas homofermentativas, ou seja, que produzem apenas um tipo de isômero (L ou D), enquanto a rota química produz uma mistura racêmica de ácido. Além do mais, podem-se reduzir os custos de produção quando utilizados substratos de baixo custo no meio fermentativo, como resíduos agro-industriais; a seleção de resíduos que dispensam a etapa de hidrólise adiciona economia ao processo. Nesse sentido, esforços têm sido dirigidos no intuito de descobrir novas linhagens microbianas, que sejam eficientes em produzir níveis elevados de apenas um isômero de ácido lático, empregando-se substratos alternativos e de baixo custo.

O Brasil é um país que domina a produção de muitas culturas, com acentuado crescimento no setor de agronegócios, e geralmente o processamento industrial dessas matérias primas geram diferentes tipos de resíduos agro-industriais, que podem ser utilizados como fonte de carbono e nitrogênio nos processos fermentativos.

A produção de ácido lático tem sido reportada por diversos micro-organismos, incluindo bactérias, fungos filamentosos e leveduras (ILMÉN et al., 2013; SUN e ZHU, 2012, DING e TAN 2006). As bactérias láticas são as mais utilizadas, e diferentes processos fermentativos têm sido reportados. Cada linhagem de micro-organismo apresenta atividade metabólica em faixas específicas ótimas de pH e temperatura, que influenciam em fatores como atividade enzimática e permeabilidade da membrana microbiana, qual facilita a passagem dos nutrientes do meio e a saída de produtos, por exemplo. Outros parâmetros do processo fermentativo podem influenciar no desempenho dos micro-organismos; a agitação está intimamente relacionada com a homogeneidade do meio de cultivo, promovendo um

maior contato das células com os componentes do meio; por outro lado determinadas velocidades de agitação podem causar cisalhamento, assim como interferir no oxigênio disponível no sistema. A concentração de substrato também tem grande influência no processo. Elevadas concentrações de fonte de carbono podem causar repressão catabólica, inibindo a produção, enquanto baixas concentrações de substrato obviamente levam à conversão de pequenas quantidades de produto formado. As técnicas de bateladas alimentadas vêm solucionar esse impasse, uma vez que pode se manter uma baixa concentração de substrato no interior do reator, adicionando-se estrategicamente o substrato conforme a necessidade. Existem várias estratégias de alimentação, desde as mais simples como pulsos que podem ser aplicados manualmente, como as mais elaboradas a partir de softwares, onde são inseridos dados referentes à cinética fermentativa do micro-organismo utilizado, e o programa estima a quantidade necessária de suprimento de substrato necessário para o crescimento microbiano e a conversão em produto.

Durante a fermentação, o ácido lático formado é responsável por baixar o pH do meio, o que pode interferir no metabolismo do micro-organismo, por isso se faz necessário o controle do pH por algum agente neutralizante (HONGO et al. 1986).. Vários controladores de pH podem ser utilizados, e a escolha deste no processo é muito importante, uma vez que o controlador associa-se ao ácido lático transformando-o em lactato, o tipo de lactato formado direcionará os processos subsequentes de purificação do ácido lático. O agente controlador de pH também esta intimamente relacionado com os custos do processo.

O processo de *downstream* é uma etapa muito importante por apresentar expressiva contribuição referente aos custos finais de obtenção do produto, assim como a geração de resíduos. Diferentes técnicas de separação e purificação do ácido lático têm sido reportadas dependendo da constituição do meio fermentado final (GONZALEZ et al., 2006; TIMBUNTAM et al., 2008). GONZALEZ et al., 2006; TIMBUNTAM et al., 2008;.

Diante da expressiva aplicabilidade industrial do D(-) ácido lático e da necessidade de se desenvolver processos de produção economicamente competitivos e ambientalmente amigáveis, este estudo tem como objetivo estabelecer as melhores condições para a produção de D(-) ácido lático pelos micro-organismos *Sporolactobacillus nakayamae* e *Lactobacillus delbrueckii* através de modificações no meio de cultivo e condições de fermentação.

2 OBJETIVOS

2.1 **Objetivo geral**:

Desenvolver processos fermentativos utilizando recursos de baixo custo, onde os micro-organismos *S.nakayamae* e *L. delbrueckii* sejam capazes de produzir elevados níveis de D(-) ácido lático.

2.2 Objetivos específicos:

- Selecionar fontes de carbono e nitrogênio que induzam eficiente produção de D(-) ácido lático pelos micro-organismos;

- Verificar quais componentes do meio de cultivo são significativos na produção de ácido lático, assim como as concentrações apropriadas dos mesmos para o processo fermentativo, utilizando planejamentos experimentais;

- Avaliar qual o agente neutralizante de pH mais apropriado para cada processo;

- Estudar a influência das condições de cultivo como pH, temperatura, agitação e tamanho de inóculo na produção de D(-) ácido lático por fermentação em batelada;

- Avaliar diferentes estratégias de alimentação em batelada alimentada;

- Estudar a extração e purificação do ácido lático do meio fermentado através de métodos alternativos àqueles utilizados na indústria.

3 Revisão da Literatura

3.1 O ácido lático

O ácido lático (ácido 2-hidroxipropanóico), $CH_3CHOHCOOH$ é um ácido orgânico, uma das menores moléculas opticamente ativas, o qual tem sido extensivamente utilizado em todo o mundo, em uma variedade de aplicações industriais e biotecnológicas. A existência de um carbono assimétrico na posição alfa da função ácida é a fonte de duas formas enantioméricas desta molécula, chamados, respectivamente, L(+) e D(-) (Figura 1) (GHAFFAR et al., 2014; SODERGARD e STOLT, 2002).

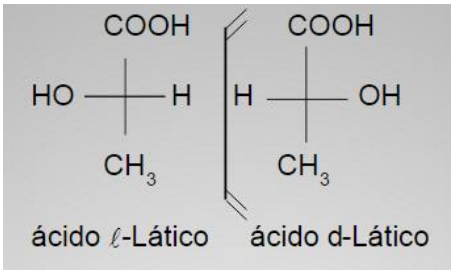

Figura 1. Estruturas químicas de Fischer dos enantiômeros de ácido lático

O ácido lático pode ser obtido quimicamente ou por fermentação microbiana. A produção por fermentação resulta na formação de D(-) ou L(+) ácido lático, ou uma mistura racêmica, dependendo do micro-organismo utilizado. Métodos homofermentativos são preferencialmente utilizados na produção industrial, uma vez que essa via resulta em alto rendimento do produto e pequena formação de sub-produtos (JOHN e NAMPOOTHIRI, 2006; MEHTA et al. 2007). A maioria das bactérias produtoras de ácido lático sintetiza as duas formas dos estereoisômeros, contudo, apenas algumas têm a capacidade de produzir um isômero de elevada pureza óptica. Na última etapa da fermentação lática, o ácido pirúvico é convertido a ácido lático pela atividade da enzima lactato desidrogenase (LDH) qual é dependente de NAD+. Cada isômero é formado por uma LDH específica, a L-lactato desidrogenase (L-LDH) está relacionada com a conversão do isômero L(+), enquanto o D(-) ácido lático é produzido pela D-lactato desidrogenase (D-LDH) (STOCK et al., 1997). Algumas bactérias apresentam a lactato racemase (LR), responsável pela conversão de um isômero (D(-) em L(+)) e vice versa. Além disso, as enzimas responsáveis pela determinação de isomeria do ácido lático produzido são expressas com intensidades diferentes dependendo de características genéticas dos micro-organismos. (GARVIE, 1980).

Isômeros puros, L e D ácido lático apresentam aplicação industrial específica, a partir da polimerização desses monômeros diferentes tipos de polímeros de ácido lático (PLA) são formados. As propriedades do PLA dependem da proporção dos enantiômeros, o que permite a produção de polímeros com diferentes características, voltadas para aplicações específicas (AURAS et al., 2003). Portanto, muitos estudos visam obter a forma pura dos isômeros D(-) (TANAKA et al., 2006; LU et al., 2009) ou L (+) (WEE et al. 2006b; QIN, et al. 2009; LIMA et al., 2011).

Alguns estudos envolvendo engenharia metabólica trabalham no direcionamento da produção de apenas um isômero, silenciando uma LDH específica a fim de obter produção de um isômero específico, de alta pureza óptica (OKANO et al., 2010; ASSAVASIRIJINDA et al. 2016).

As bactérias ácido láticas são capazes de fermentar o açúcar por diferentes vias resultando em um processo homo ou hetero fermentativo. A via homofermentativa resulta apenas em ácido lático como produto final do metabolismo da glicose, pela via Embden–Meyerhof–Parnas (Figura 2a), já na via heterofermentativa quantidades equimolares de ácido lático, dióxido de carbono e etanol ou acetato são formadas a partir da glicose, por via pentose-fosfato (figura 2b) (LEHNINGER et al., 2002)

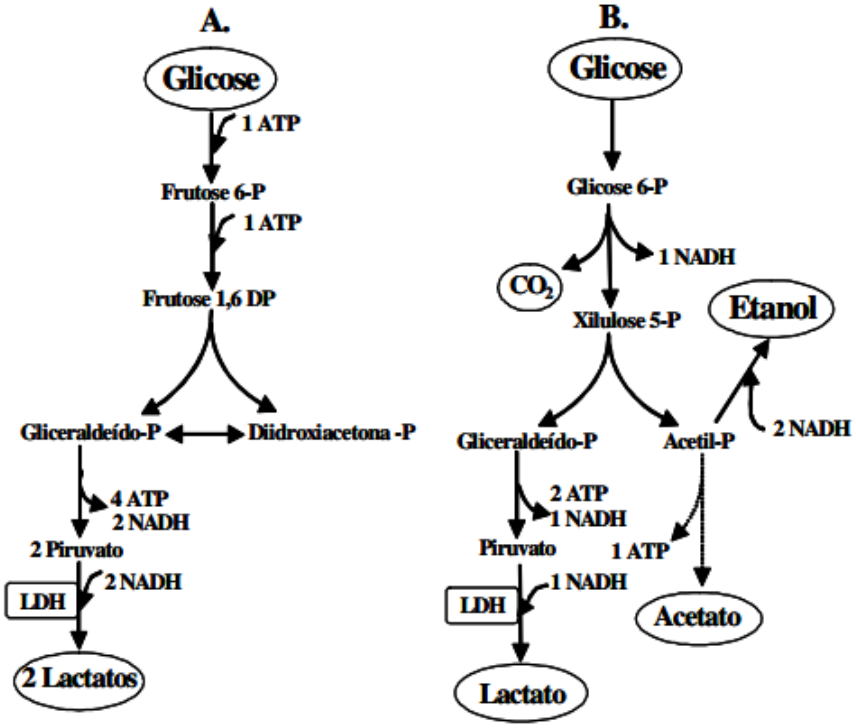

Figura 2. Vias catabólicas das bactérias ácido láticas: a) Homofermentativa B) Heterofermentativa. Adaptado de HOFVENDAHL e HÄGERDAL, (2000).

Embora a tecnologia de fermentação em grande escala para a produção de L(+) ácido lático já esteja há muito tempo bem estabelecida, os processos que visam economia e eficiência para produção de D(-) ácido lático em escala industrial, ainda devem ser aprimorados. (HOFVENDAHL e HAHN-HAGERDAL, 2000; JOHN et al., 2007). Segundo Liu et al., (2014), para obter informações relevantes relacionadas à um processo de produção de D(-) em larga escala, é necessário demonstrar uma fermentação eficiente em escala piloto, utilizando uma linhagem microbiana promissora, com meio de cultivo e neutralizadores de pH de baixo custo.

No início da década de 1960, foi desenvolvido um método de síntese química do ácido lático, considerando o interesse na indústria de panificação. A manufatura do ácido lático por via sintética em escala comercial teve início em 1963 no Japão e Estados Unidos (HOLTEN, 1971; VICKROY, 1985). Na síntese química, a lactonitrila é produzida através da combinação de cianeto de hidrogênio e acetaldeído, na presença de um catalisador, em fase líquida. Em seguida, a lactonitrila é hidrolisada em ácido lático, por ácido sulfúrico ou clorídrico. Neste processo cloreto de amônio é gerado como sub-produto (PAL e DEY, 2012). Contudo, a síntese por via química resulta em uma mistura de D(-) e L(+) ácido lático, além do mais, não é um processo economicamente viável (WEE et al. 2006a).

Em comparação com a síntese química, o processo biotecnológico para produção de ácido láctico oferece várias vantagens, como, baixo custo de substrato, temperatura de produção e consumo de energia (DATTA e HENRY, 2006). Na figura 3 é apresentado um esquema com as principais etapas na produção de ácido lático por via química e fermentativa.

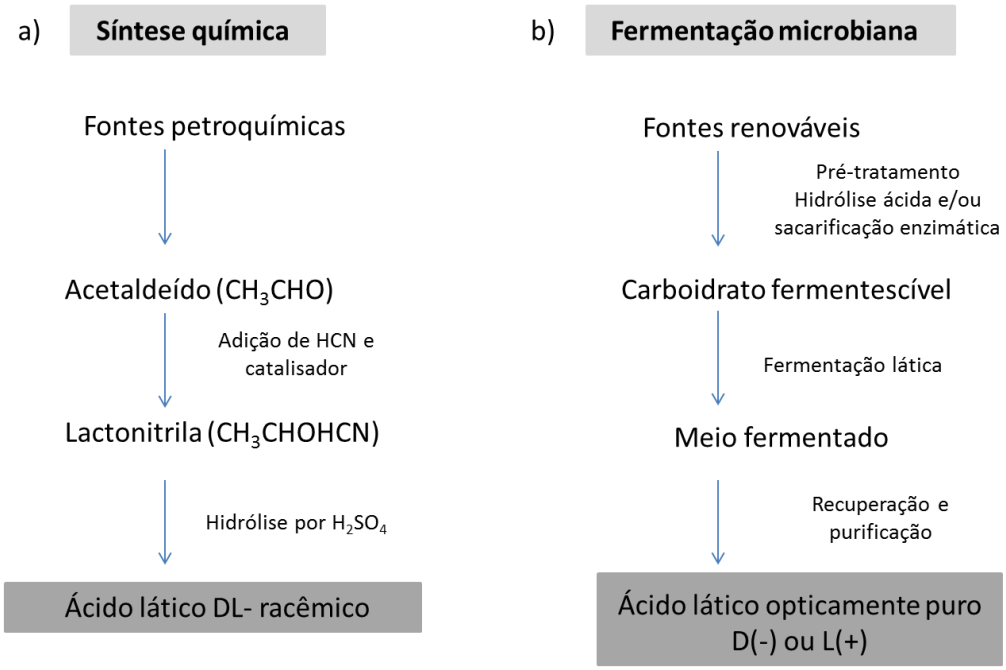

Figura 3. Métodos de produção de ácido lático a) Síntese química e b) Fermentação microbiana. Adaptado de WEE et al., (2006a).

O número de investigações envolvendo a produção de ácido lático por fermentação tem aumentado exponencialmente desde a década de 90 até os dias atuais (Figura 4). O ácido lático ocupa a terceira posição entre as 30 moléculas mais estudadas de 2016, ficando atrás dos açúcares C6 e dos Polihidroxialcanoatos (BiofuelsDigest, 2016).

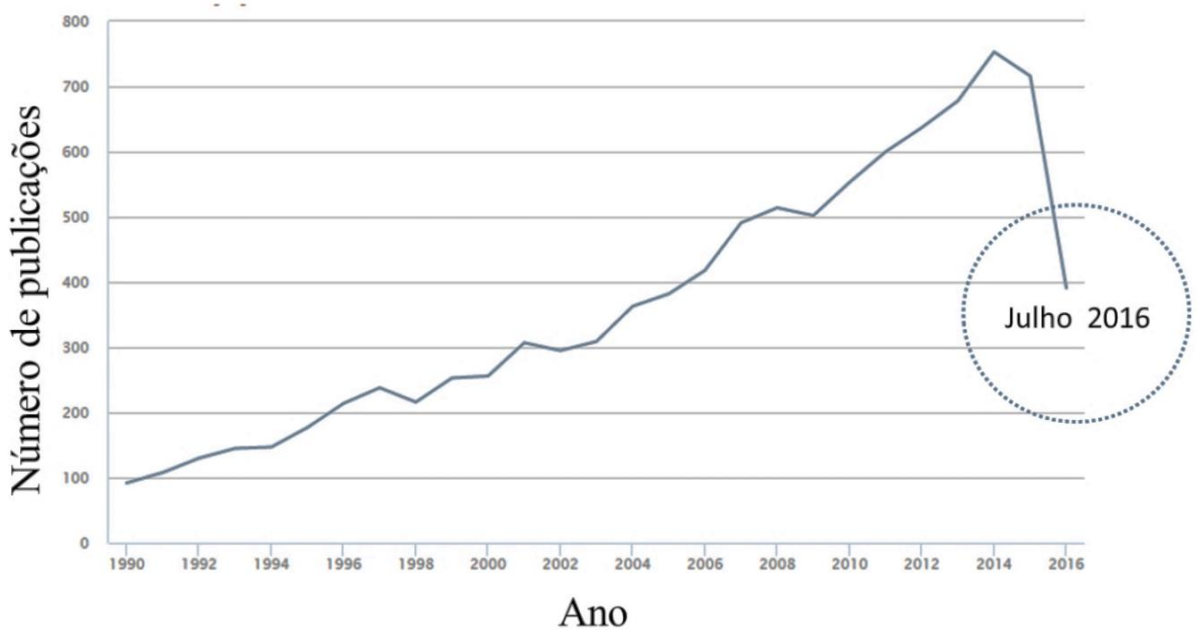

Figura 4. . Número de publicações relacionadas com a produção de ácido lático via fermentativa. Fonte: SCOPUS (2016).

A produção mundial de ácido lático em 2006 foi estimada entre 130.000 e 150.000 toneladas métricas por ano, com estimativa de um crescimento de 19% ao ano, principalmente devido ao seu emprego na produção de polímeros biodegradáveis como o ácido polilático (PLA) (WEE et al., 2006a). Em 2009, a produção mundial foi de 258.000 toneladas (GLOBAL INDUSTRY ANALYSTS INC, 2011). O maior produtor, *NatureWorks*, apresentou produção de 140 mil toneladas de PLA em 2011. Já em 2013, a produção mundial de ácido lático (D, L e DL) foi de 284.000 toneladas (DAMMER et al, 2013). Um crescimento de 329.000 toneladas foi projetado para o ano de 2015 (GLOBAL INDUSTRY ANALYSTS INC, 2011), e a produção estimada de ácido lático para 2017 é de 367.300 toneladas (ABDEL-RAHMAN et al., 2013). A previsão de mercado do ácido lático pela *Credence Research* (2016) apresenta uma estimativa anual do crescimento a partir de 2014 até 2022, baseados em informações de domínio público reportadas por algumas empresas produtoras (Figura 5).

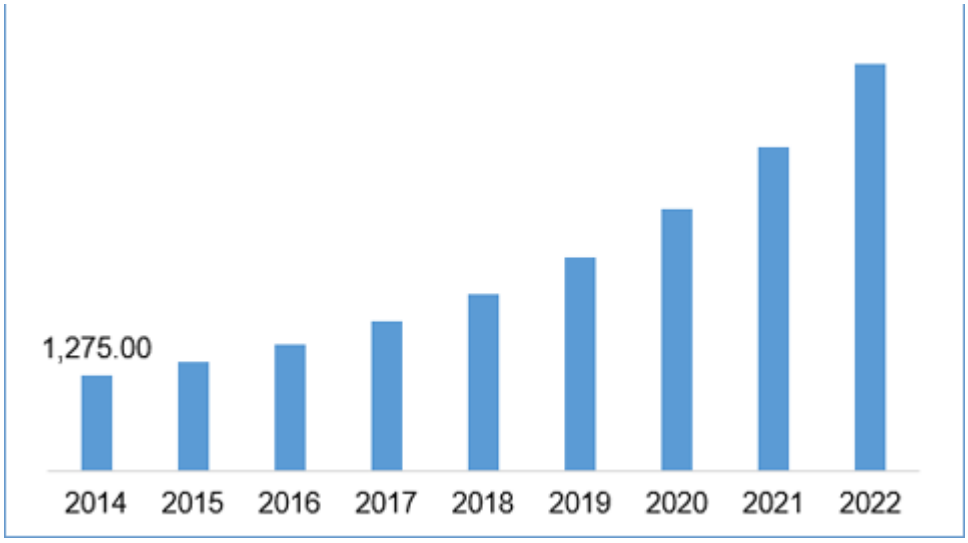

Figura 5. Previsão de Rendimento Global do mercado de Ácido lático, 2014-2022 (USD Milhões).

3.2 Polimerização do ácido lático

Existem três vias para produção de PLA a partir do ácido lático (Figura 6) (HU et al., 2016). Dentre esses métodos, atualmente, a polimerização direta e polimerização por abertura de anel do lactídio são as técnicas mais utilizadas (LASPRILLA et al., 2012; THOMAZ, 2010; GARLOTTA, 2001).

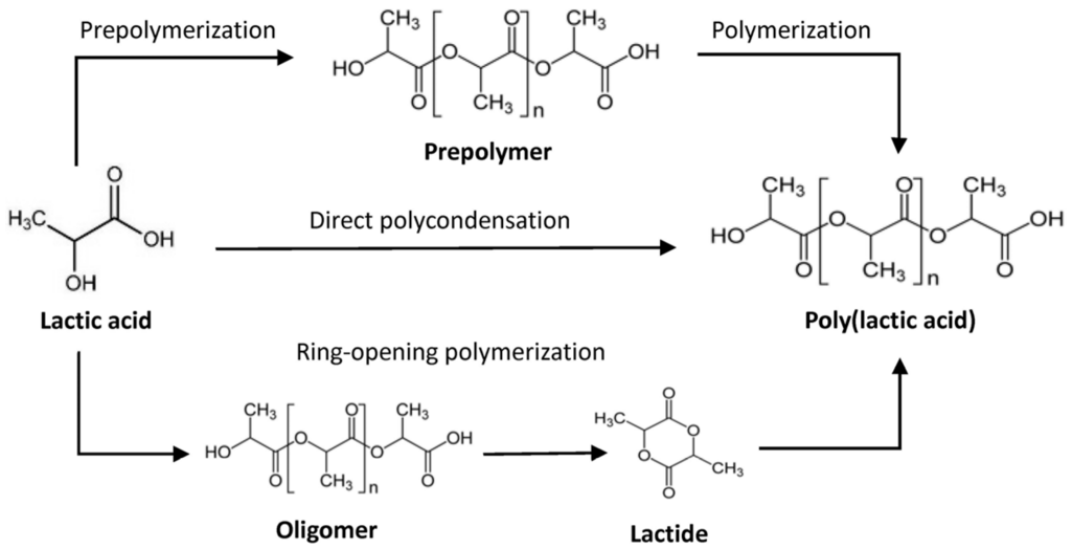

Figura 6. Vias de síntese de PLA.

O grau de cristalinidade e, muitas outras propriedades importantes, como resistência mecânica e ponto de fusão, são controladas pela razão dos enantiômeros D(-) e L(+) usados e a sequência de arranjo dos enantiômeros nos polímeros (SODEGARD e STOLT, 2002; AURAS 2010). L(+) ácido lático é usado para a síntese do poli L-ácido lático (PLLA), um polímero semi-cristalino, biodegradável e termoestável, com potencial de aplicação no mercado de embalagens. PLLA apresenta alta resistência à tração e alta capacidade de modulagem, o que o torna adequado para aplicação em produtos médicos utilizados em fixação ortopédica (pinos e ligamentos), aplicações cardiovasculares (stents, enxertos), aplicações dentárias, intestinais e suturas (JOHN et al., 2006).

O polímero constituído dos dois isômeros, chamado de poli DL-ácido lático (PDLLA) é degradado mais rapidamente, devido a sua estrutura amorfa (CHEN et al., 2003). Elevada quantidade de estereoisômeros são necessários para produção de PLLA e PDLA a fim de se obter elevada propriedade térmica no polímero. A síntese de PLLA tem sido frequentemente reportada, enquanto a produção de PDLA é raramente encontrada, devido ao custo elevado do D- Lactídio (PIVSA-ART et al., 2013)

O lactidio é um diéster cíclico do ácido láctico, e apresenta-se sob três formas: L-lactidio, D-lactidio e o meso-lactidio, que é opticamente inativo e contém uma unidade L e uma unidade D no anel (MOTTA, 2002). Neste processo de polimerização, duas moléculas de ácido lático passam por um processo de esterificação e ciclização catalítica para formar um éster de dilactídeo cíclico. Esse processo de dimerização gera água, que pode ser separada antes da polimerização, devido a uma queda significativa na polaridade. O PLA de alta massa molar é produzido a partir do lactídeo, pela reação de polimerização por abertura do anel,

usando, por exemplo, o catalisador octoato de estanho (SnOct). Este mecanismo não gera molécula de água adicional e, portanto, é possível obter uma vasta gama de massas molares (SODEGARD e STOLT, 2002).

A indústria tem exigido um processo de polimerização mais eficiente, de preferência por uma reação de um passo com um menor consumo de energia e tempo de reação mais curto (NAGAHATA, 2007). Kéki e colaboradores (2001) reportaram que a irradiação por micro-ondas acelerou o tempo de reação de polimerização. De acordo Nikolic e colaboradores (2010) as dificuldades de síntese do PLA podem ser superados com sucesso pelo aquecimento por micro-ondas, pois a radiação de micro-ondas tem numerosas vantagens quando comparado com o aquecimento convencional: aquecimento homogêneo de todo o volume da mistura de reação e transferência de energia elevada por unidade de tempo, podendo assim melhorar o rendimento, aumentar a velocidade da reação e possibilitar uma síntese sem utilizar grandes quantidades de solvente.

3.3 Aplicações biotecnológicas do ácido lático e seu polímero (PLA)

O ácido lático é um produto amplamente empregado nos mais diversos setores da indústria, como o têxtil, farmacêutica, química, alimentícia entre outros (WEE et al., 2004; ALTAF et al., 2006). A maior aplicação do ácido lático é voltada para produção de polímeros (Figura 7).

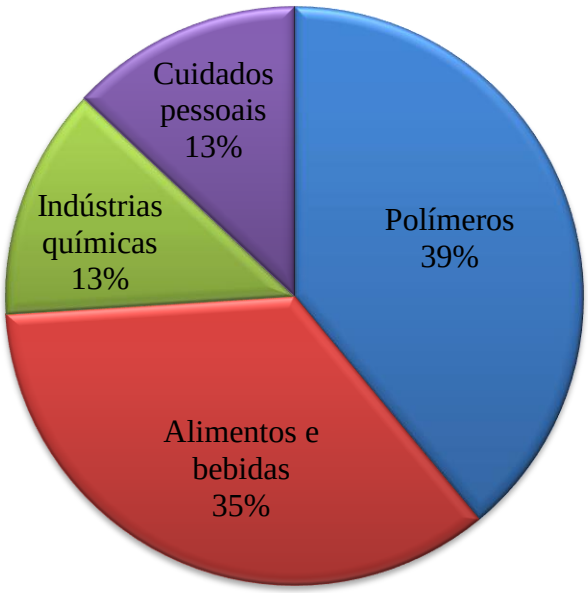

Figura 7. Aplicações biotecnológicas do ácido lático (THE ESSENTIAL CHEMICAL INDUSTRY, 2013).

O PLA tem sido considerado um dos plásticos biodegradáveis mais promissores por apresentar propriedades mecânicas comparáveis com as dos polímeros provenientes de fontes fósseis, como, elevado módulo de elasticidade, rigidez, transparência, comportamento termoplástico, biocompatibilidade e boa capacidade de moldagem (LIM, et al., 2008; LIU, et al., 2011; ZHANG e SUN, 2005). Há muito tempo os plásticos se tornaram importantes no cotidiano da sociedade, pelas características de versatilidade, resistência mecânica, durabilidade e resistência à degradação. No entanto, estas propriedades consideradas desejáveis, atualmente são consideradas preocupantes, e o acúmulo de plásticos no meio ambiente tornou-se um problema mundial (KHANNA e SRIVASTAVA, 2005). A crescente utilização de plásticos de degradação lenta colaborou com os problemas referentes à poluição. O desenvolvimento sustentável requer novos materiais com propriedades que colaborem para evitar a ocorrência desses problemas (BRITO, et al., 2011).

O custo de produção de biopolímeros é a maior limitação na substituição destes pelos polímeros sintéticos. Contudo, a utilização de produtos elaborados a partir de biopolímeros apresenta-se de forma crescentes no mercado (Figura 8).

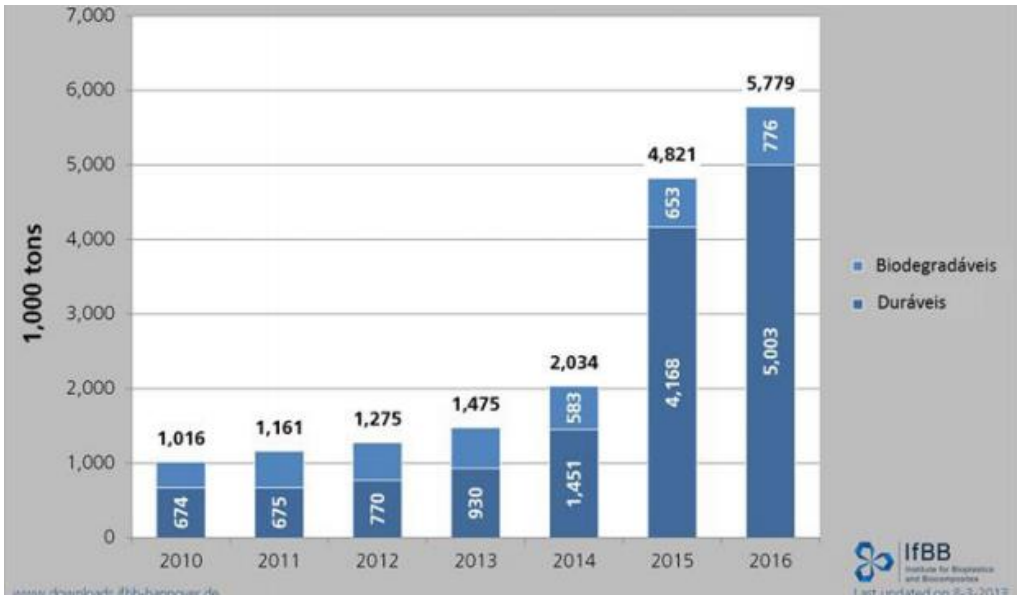

Figura 8. Demanda global de produção de biopolímero Fonte: INSTITUTE FOR BIOPLASTICS AND BIOCOMPOSITES, 2016.

O desenvolvimento de polímeros biodegradáveis e bio-reabsorviveis têm ganhado atenção nos últimos anos pela sua ampla aplicação ambiental e médica. O PLA é um poliéster

alifático que ocupa posição entre os mais importantes polímeros (Figura 9) (ASLAN et al., 2000).

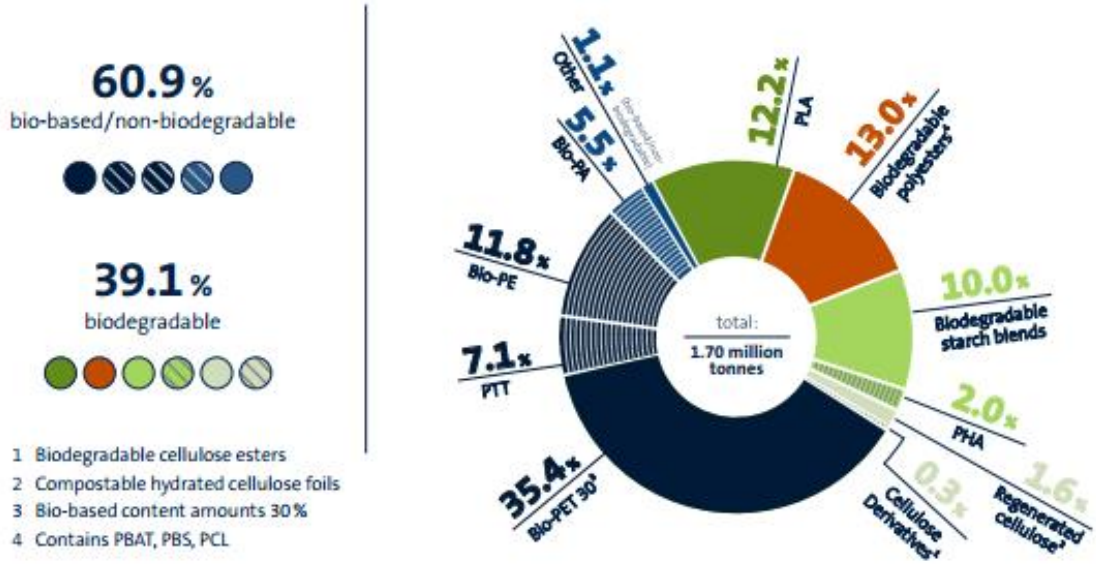

Figura 9. Capacidade de produção de Bioplástico. Fonte: Institute for Bioplastic and Biocomposites, 2016.

A medicina tem sido uma das áreas com maior interesse no emprego do PLA. Os polímeros de ácido lático têm a importante característica de ser bioreabsorvíveis, podendo ser empregados na regeneração de tecidos, suturas, fixações de fraturas, reposição óssea, reparo da cartilagem, reparo do menisco, fixação de ligamentos e implantes; também vêm sendo produzidos parafusos, próteses, pinos, grampos e placas de aplicações ortopédicas e cirurgias orais em humanos e animais (SAKATA et al., 2004; GOLLWITZER et al., 2005). O homopolímero de ácido lático apresenta característica cristalina, a qual esta associada com um período longo de degradação. Quando combinados D(-) e L(+) na produção de um polímero, a taxa de degradação é mais elevada, podendo-se melhorar as propriedades mecânicas, proporcionando um tempo de degradação adequado, o que o torna promissor para aplicações médicas (MOTTA, 2006). Pinos e parafusos para ossos assim como placas ósseas a partir de PLA são promissores substitutos de implantes metálicos. A característica de biocompatibilidade e bio-reabsroção somam vantagens para a utilização deste material, principalmente por dispensar o procedimento de posterior remoção dos implantes (LU, 2000; KULKARNI et al., 1971).

Na indústria alimentícia o ácido lático é empregado como preservativo, acidulante, flavorizante e emulsificante, além de inibir a esporulação de bactérias em alimentos processados (LI et al., 2004; NAVEENA et al., 2005). O L(+) ácido lático é preferido nas

indústrias alimentícias e farmacêuticas, uma vez que os seres-humanos apresentam apenas a enzima LLDH, o D(-) ácido lático pode ser prejudicial para o metabolismo humano (ADNAN e TAN, 2007).

3.4 Fatores que influenciam na produção de ácido lático

A utilização de um micro-organismo em um processo biotecnológico envolve geralmente a produção de algum composto de interesse. No entanto, as espécies que são encontradas na natureza normalmente não produzem grandes quantidades de metabólitos, somente o necessário para a sobrevivência. Dessa forma, vários trabalhos visam melhorar essa produção para que seja aplicável em escala industrial (PAREKH, 2000).

Parâmetros operacionais são controlados a fim de otimizar o processo de fermentação, diversos são os fatores que influenciam na produção de ácido lático por bactérias ácido-láticas, como por exemplo, pH (MUSSATTO et al., 2008; ANDERSEN et al., 2009), temperatura (TANGO e GHALY, 1999), aeração (OKINO et al. 2008) fontes de carbono (BULUT et al. 2004), fontes de nitrogênio (NANCIB, et al. 2001; ALTAF et al. 2007), sais minerais (HAULY, 2001), vitaminas (XU et al. 2008) entre outros.

Os micro-organismos apresentam faixas de pH e temperatura ótimos para seu crescimento, que varia conforme a espécie ou linhagem. Estudos demonstram que o controle do pH durante a fermentação favorece a produção de ácido lático (MUSSATTO et al., 2008). O pH extracelular é um dos principais fatores relacionados com a produção de ácido lático influenciando a atividade catalítica das enzimas. O pH ótimo para produção de ácido lático varia entre 5.0 e 7.0, linhagens de *Lactobacillus* apresentam pH ótimo em torno de 5.7, são conhecidas como ácido tolerantes (KASHKET, 1987;SILVA e MANCILHA, 1991). Muitas bactérias são sensíveis a pHs ácidos, e esta sensibilidade forneceu a base para a conservação de alimentos e manipulação de fermentações industriais (MILLER e WOLIN. 1981). O pH tem efeito até mesmo no direcionamento da via fermentativa do micro-organismo, como reportado por Russell e Hino (1985), utilizando *Streptococcus bovis* apresentou produção de formato, acetato e etanol em pH neutro, porém em pH extracelular baixo apresentou fermentação homolática (RUSSELL e HINO, 1985). O mesmo foi reportado por Stokes (1949) estudando o comportamento da *Escherichia coli,* onde verificou que, em pH ácido diminuiu a produção de acetato, formato e etanol e aumentou a produção de ácido lático. A mudança de pH também pode influenciar no crescimento celular, como reportado por Fiedler et al., (2011), em sua pesquisa observaram que a alteração do pH esta relacionado com o rendimento de massa celular produzida por mol de ATP em *Streptococcus*

pyogenes e *Lactobacillus lactis,* maiores rendimentos foram verificados em pH próximo ao habitat natural dos micro-organismos.

A temperatura é um fator ambiental, qualquer variação exerce efeito sobre o curso da fermentação, reações catalisadas por enzimas são especialmente sensíveis a pequenas mudanças na temperatura (MERRITT, 1966). As espécies de micro-organismos apresentam temperaturas ótimas distintas, que estão relacionadas com o crescimento celular e produção de ácido lático, portanto é conveniente estudar qual a temperatura apropriada no processo de fermentação para cada caso (PELEG, 1995). Quando um micro-organismo é submetido a temperaturas diferentes daquelas consideradas ideais, ocorre inibição do crescimento e em alguns casos destruição dos produtos (SCHEPER et al., 2000).

A agitação está relacionada à homogeneização do meio, à dissipação do calor produzido em decorrência das reações metabólicas, transferência de calor para o controle da temperatura, assim como minimizar a morte celular resultante da adição de ácido e base concentrados para o controle de pH (CHARLES, 1985).

3.5 Meio de cultivo para fermentação lática: Fontes alternativas de carbono e nitrogênio

Comportamentos sociais e hábitos de consumo marcados pelo grande desperdício de recursos naturais culminaram em problemas sociais, ambientais e econômicos, o que faz da sustentabilidade um tema rotineiramente discutido, chamando a atenção de governos, organizações e da comunidade acadêmica (MALHOTRA et al., 2013). A preocupação com o ambiente tem estimulado pesquisadores do mundo todo a desenvolver métodos para produzir uma ampla gama de moléculas e materiais por tecnologia sustentável (ABDEL-RAHMAN et al., 2013).

Os meios comerciais utilizados para o crescimento de micro-organismos fastidiosos, como bactérias láticas, não são economicamente atrativos e a fonte de carbono é um dos maiores responsáveis pelo custo do processo fermentativo (GONZÁLEZ et al., 2007). Uma alternativa é a utilização de substratos alternativos como soro de queijo (GHALY et al. 2004; KIM et al. 2006), vinhaça, melaço (WEE et al. 2004), caldo de cana (CALABIA e TOKIWA, 2007), açúcar de beterraba (KOTZAMANIDIS et al. 2002), óleo de soja hidrolisado (KWON et al. 2000), resíduo de óleo, amido (YUMOTO e IKEDA, 2004; OHKOUCHI e INOUE, 2006), amido de arroz (FUKUSHIMA et al. 2004), água de manipueira (COELHO, et al., 2010) bem como diversos produtos e resíduos agrícolas (NARAYANAN et al., 2004,

ZHANG et al. 2007). As fontes alternativas complexas usadas para diminuir os custos de produção, têm substâncias ou características físicas que podem influenciar na produção do ácido lático e até mesmo no crescimento do micro-organismo utilizado (OHKOUCHI e INOUE, 2006).

Estudos têm reportado produção de ácido lático através de conversão de biomassa (TOKUHIRO et al. 2008). Entretanto, para tornar acessíveis os açúcares da biomassa são necessárias operações como pré-tratamento químico e hidrólise enzimática da biomassa lignocelulósica, ou sacarificação enzimática de biomassa amiláceas (LI e CUI, 2009). Nos pré-tratamentos tradicionais de biomassa lignocelulósica, são adicionados produtos químicos como ácidos e bases, que terão influência na etapa final de separação e purificação do produto, além de adicionar custo ao processo (HOFVENDALH e HAGERDAL-HAHN, 2000). Contudo, o custo de produção de ácido láctico pode ser significativamente reduzido quando se utilizam sub-produtos de outros processos, tais como soro de leite ou melaço de cana-de-açúcar contendo açúcares prontamente fermentáveis (DUMBREPATIL, 2008).

O soro de leite é um subproduto da fabricação de queijo, rico em proteínas, gorduras e lactose de excelentes propriedades funcionais, nutricionais e tecnológicas, (PELEGRINE e CARRASQUEIRA, 2008). O melaço é um material viscoso considerado subproduto da produção de açúcar, composto de sacarose, glicose e frutose, apresentando concentração total de carboidratos de 45-60% (MARIAM, et al 2009).

Entre as fontes alternativas de nitrogênio reportadas estão: farinha de amendoim (WANG et al., 2011), farinha de soja (KWON et al. 2000), proflo, água de maceração de milho (YU et al., 2008), autolizado de levedura (LIMA et al., 2009, COELHO et al. 2011) , entre outras.

O proflo se trata de um pó fino amarelo produzido a partir de sementes de algodão. É rico em proteína (56% p /v), e contém 24% de hidratos de carbono, 5% de óleo e 4% de cinzas, sendo esta última rica em cálcio, ferro e cloreto de fósforo (OKAFOR, 2007). A água de maceração de milho é uma alternativa muito econômica em relação às fontes de nitrogênio mais puras como extrato de levedura e peptona. Este resíduo é um subproduto proveniente do processamento do amido de milho (WEE et al., 2006b), sendo o nitrogênio disponibilizado na forma de aminoácidos como alanina, arginina, ácido aspártico, cistina, ácido glutâmico, histidina, iso-leucina, leucina, lisina, metionina, fenilalanina, prolina, treonina, tirosina e valina, também estão presentes algumas vitaminas do complexo B (CARDINAL e HEDRICK, 1948).

A produção em larga escala do PLA tem sido dificultada pelos altos custos, principalmente pelo valor elevado do ácido lático. Dessa forma, a redução dos custos na produção, assim como o desenvolvimento de processos fermentativos eficientes que resultem em alta produtividade, são objetivos de estudo atuais. (ABDEL-RAHMAN et al. 2013). Para tanto, continuamente têm se investigado diversos substratos de baixo custo que sejam capazes de serem convertidos em ácido lático por micro-organismos.

3.6 Produção do ácido lático por fermentação

O ácido lático tem sido produzido por vários tipos de fermentações, tais como, fermentação submersa e em estado sólido, fermentação contínua com sacarificação simultânea, processos contínuos com recirculação celular, fermentação por batelada, batelada alimentada entre outros (MARQUES et al. 2008, COELHO et al., 2011b).

Dentre os diversos tipos de biorreatores empregados nos processos biotecnológicos, o mais utilizado é o tanque agitado em processos submersos, correspondendo a 90% do total de biorreatores utilizados industrialmente. A primeira aplicação deste processo foi durante a segunda guerra mundial para a produção de insulina (BAILEY, 1980). A fermentação submersa permite uma melhor dissolução dos nutrientes do meio, facilitando o contato destes com os micro-organismos, assim como o controle da temperatura e pH durante a fermentação (SCHMIDELL et al., 2001).

O processo em batelada é o mais utilizado, porém apresenta baixa produtividade devido ao longo tempo de fermentação, assim como baixas concentrações celulares, devido à inibição pelo produto final (ABDEL-RAHMAN et al, 2013). Um fator importante a ser considerado nos processos fermentativos em batelada é repressão catabólica pelo substrato. Muitas vezes a taxa de conversão do substrato em produto pelo micro-organismo é alta, sendo o substrato inicial consumido em um curto período de tempo, cessando a fermentação. Contudo, uma concentração elevada de substrato inicial pode levar à inibição da produção. A repressão catabólica pode ser evitada mantendo uma baixa concentração de substrato no interior do reator, provendo quantidades não inibitórias ao longo do processo, conforme requerido, o que caracteriza o processo como batelada alimentada. Vários estudos utilizam esta técnica com o propósito de evitar a inibição da produção pelo substrato e, por conseguinte, elevar os níveis de produção e produtividade (ABDEL-RAHMAN et al. 2015).

Diferentes estratégias de alimentação podem ser utilizadas nas bateladas alimentadas, como a alimentação por pulsos, contínua, pH-Stat e exponencial. Vários estudos têm reportado melhora na produção de ácido lático quando utilizada a estratégia de alimentação

por pulsos (SON e KWON, 2013; WANG et al., 2011; MENG et al., 2012). Nesta técnica determinadas concentrações de substrato são inseridas uma ou mais vezes no reator, em intervalos determinados conforme a necessidade.

Ding e Tan (2006) estudaram os efeitos de diferentes estratégias de alimentação para produção de L(+) ácido lático por *L. casei*, e de acordo com seus resultados a alimentação exponencial foi o método mais eficiente, aumentando a produção de ácido lático em 56,5%, em comparação com o processo em batelada. Na alimentação exponencial podem ser utilizados softwares onde são inseridas fórmulas específicas com os valores referentes à cinética do processo fermentativo do micro-organismo em estudo. Esses programas são capazes de ajustar o fluxo da alimentação conforme a necessidade, levando em consideração as taxas de conversão, assim como a velocidade em que as mesmas ocorrem.

Zhang et al., (2010) aplicaram a técnica de pH-Stat na produção de ácido lático por *L. lactis*, nesta estratégia a alimentação é baseada no controle do pH, considerando uma relação linear entre o consumo da base utilizada para o controle de pH, e utilização do substrato durante a fermentação lática. A base e o substrato são misturadas proporcionalmente e então a concentração do substrato é controlada através do ajuste do pH, automaticamente.

Na fermentação em batelada contínua, é provida alimentação com os nutrientes necessários continuamente, ao mesmo tempo em que são removidas as células, produtos e resíduos do processo. O volume da cultura é mantido constante, assim como as concentrações de nutrientes. Não é um processo preferido no setor industrial pelo alto risco de contaminação e mutação do micro-organismo produtor (SCHMIDELL, 2001).

Lu et al., (2012) estudaram um processo fermentativo em reator de escala piloto equipado com sistema de micro-filtração para o reciclo de células, e alimentação por pulsos, neste processo as células foram recicladas 12 vezes, segundo os autores o método se mostrou eficiente e promissor para ser utilizado em escala industrial.

Alguns estudos reportam produção de ácido lático utilizando células imobilizadas a fim de aumentar a densidade celular do processo, porém não são reportados resultados promissores relacionados com aumento de produção e produtividade, sendo reportados melhores resultados utilizando células livres (HOFVENDAHL e HAHN–HAGERDAL, 2000).

3.7 Neutralizadores de pH

Durante a fermentação lática, o acumulo de ácido lático é responsável por baixar o pH do meio, o que pode ocasionar efeitos inibitórios nas atividades metabólicas do micro-

organismo (HONGO et al. 1986). Sabe-se que o pH é o parâmetro mais importante relacionado com a estabilidade enzimática, uma queda brusca de pH resulta na diminuição das reações enzimáticas (WASEWAR et al., 2003). Dessa forma, o processo de fermentação requer um controle de pH através de agentes neutralizadores, que acabam por associar-se à molécula de ácido lático, formando lactato, podendo aumentar os custos do processo em 50%, considerando os custos dos destes agentes, assim como as etapas adicionais no processo de separação e purificação do ácido lático (EYAL e BRESSLER, 1993, WASEWAR et al.2003).

Existem vários agentes controladores de pH que podem ser utilizados nos processos fermentativos. O carbonato de cálcio e hidróxido de sódio são comumente utilizados (YEN et al., 2010; ALTAF et al. 2007; YU et al. 2007)

Nos processos industriais, quando são utilizados agentes como $Ca(OH)_2$ ou $CaCO_3$, como agentes neutralizadores, posteriormente é utilizado H_2SO_4 para precipitação, formando grande quantidade de sulfato de cálcio, considerado um resíduo sólido (ABDEL 2013; WASEWAR et al., 2003).

Porém neutralizadores como o $CaCO_3$ e $Ca(OH)_2$ têm demonstrado bons resultados nos processos fermentativos. Liu et al., 2014 reportaram um aumento de 3 vezes na produtividade utilizando $Ca(OH)_2$ em comparação com KOH e NH_4OH, em escala piloto, obtendo 126 kg/tonelada e rendimento de 97%.

Yen et al,. 2010 estudando vários agentes neutralizadores de pH na produção de ácido lático por *Rhizopus oryzae* obtiveram maior produção de ácido lático quando utilizaram $CaCO_3$ como agente neutralizador, em comparação com hidróxido de sódio (NaOH), solução de amônia e bicarbonato de sódio ($NaHCO_3$) . Outros autores obtiveram bons resultados ao utilizar solução de amônia (MARTA et al. 2003). Nakano et al., 2012, estudaram os efeitos de alguns controladores de pH como $Ca(OH)_2$, NH_4OH, e NaOH no processo de fermentação lática por *L. delbrueckii*, e verificaram maior produção de ácido lático, assim como maior crescimento celular quando $Ca(OH)_2$ foi utilizado como agente controlador. Estes autores relacionaram esses resultados com o fato da reduzida molaridade apresentada pelo lactato no meio fermentativo, evitando stress celular, resultando numa alta produtividade. Além do mais, a neutralização por cátions divalentes como Ca^{2+} apresenta-se mais eficiente se comparada com cátions monovalentes, como Na^+ e NH_4. A fim de evitar a geração de resíduos insolúveis na forma de sulfato de cálcio, Assavasirijinda et al. (2016) obtiveram bom níveis de produção de ácido lático utilizando NaOH como agente neutralizador de pH, por um *Bacillus* sp. tolerante a altas concentrações de lactato de sódio. Considerando posterior purificação por

eletrodiálise, o NaOH pode ser reutilizado no processo fermentativo, não sendo gerados resíduos (WU et al., 2011).

3.8 Micro-organismos envolvidos na produção de ácido lático

A produção de ácido lático tem sido reportada por bactérias fungos e leveduras (ILMÉN et al., 2013; SUN e ZHU, 2012, DING e TAN 2006), porém o maior número de estudos reporta tal produção por via bacteriana.

Osawa et al., 2009 reportaram uma produção de 85 g/L de L(+) ácido lático por *Candida boidinii* modificada geneticamente com alteração no gene da piruvato carboxilase a fim de reduzir a produção de etanol. O fungo filamentoso mais reportado para produção de ácido lático é o *Rhizopus oryzae* geralmente apresentando produção a partir de sub-produtos como o glicerol (VODNAR et al., 2013), resíduos como esterco (SUN e ZHU, 2012), ou resíduos ricos em amido (TASKIN et al., 2012, YEN et al., 2010).

As bactérias acido láticas têm sido tradicionalmente utilizadas para produção de ácido lático e são as candidatas predominantes para produção industrial (OKANO et al., 2010). Este grupo de bactérias caracteriza-se como gram-positivas, aeróbias ou anaeróbias facultativas, apresentam-se na forma de cocos ou bacilos, as quais produzem ácido lático por fermentação de carboidratos, são incapazes de utilizar lactato e não são patogênicas (reconhecidas como "GRAS"– Generally Recognised As Safe) (AXELSSON, 2004; HOFVENDAHL e HÄGERDAL, 2000,). Pertecem a diversos gêneros incluindo *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus*, e *Weissella* (RATTANACHAIKUNSOPON e PHUMKHACHORN, 2010; CARR et al.,2002).

3.8.1 O gênero *Sporolactobacillus*

Sporolactobacillus pertence ao filo Firmicutes, classe Bacilli, ordem Bacillales e família , Sporolactobacillaceae (NCBI, 2013).

Trata-se de uma bactéria gram-positiva produtora de esporos, micro-aerofílica e mesofílica, que produz exclusivamente D(-) ácido lático via homofermentativa. Encontrado principalmente em rizosfera de plantas selvagens (HOLZAPFEL, 1988).

Este gênero foi nomeado por Oki Nakayama, um microbiologista japonês que isolou um grande número de cepas *Sporolactobacillus* (LPSN, 2013), sendo conhecido pela capacidade de tolerância a altas concentrações de ácido lático, assim como a produção do mesmo

(ZHENG, 2011). Poucos estudos envolvendo a produção de ácido lático por este gênero têm sido reportados (tabela 1), sendo que o primeiro artigo da literatura que demonstra a produção de D(-) ácido lático pela espécie *nakayamae* é reportado nesta tese.

Tabela 1. Micro-organismos do gênero *Sporolactobacillus* envolvidos na produção de D(-) ácido lático

Micro-organismo	**Produção de D(-) ácido lático**	**Fontes de carbono e Nitrogênio**	**Referência**
Sporolactobacillus sp. CASD	207 g/L	Glicose e Farinha de amendoim	WANG et al., 2011
Sporolactobacillus nakayamae	122,41g/l	Açúcar cristal e extrato de levedura	Neste estudo
Sporolactobacillus nakayamae	112,93 g/L	Açúcar cristal e Farinha de amendoim	BEITEL et al., 2016
Sporolactobacillus inulinus ATCC 15538	93,4 g/l	Glicose e extrato de levedura	ZHENG et al., 2011
Sporolactobacillus sp. strain CASD	90,00	Glicose e extrato de levedura	ZHAO et al., 2010

3.8.2 O gênero *Lactobacillus*

De acordo com Garrity et al., (2004), as bactéria do gênero *Lactobacillus* pertencem a família Lactobacillaceae, ordem Lactobacillales, classe Bacilli e filo Firmicutes. Os lactobacilos são encontrados em substratos ricos em carboidratos, em alimentos fermentados, na mucosa de mamíferos e em plantas (HAMMES e VOGEL, 1995). Algumas espécies deste gênero como *Lactobacillus rhamnosus* são capazes de produzir L(+) ácido lático, enquanto outras têm o potencial de produzir o isômero D(-), como *Lactobacillus delbrueckii* (HOFVENDAHl e HAHN-HÄGERDAL 2000)

As bactérias da espécie *delbrueckii* são caracterizadas como gram positivas, apresentam-se na forma de bacilos, anaeróbicas facultativas, sem motilidade e sem formação de espóros. *L. delbrueckii*, faz parte do grupo das bactérias ácido láticas, portanto é ácido tolerante e possui metabolismo estritamente fermentativo sendo o ácido lático o principal produto final (AXELSSON, 1998; KANDLER e WEISS, 1986). Estudos têm demonstrado

produção de ácido lático por diversas linhagens de *L. delbrueckii* a partir de diferentes substratos, assim como por outras espécies do gênero *Lactobacillus* (Tabela 2).

Tabela 2. Micro-organismos do gênero *Lactobacillus* envolvidos na produção de ácido lático

Micro-organismo	**Isômero**	**Produção de ácido lático**	**Fontes de carbono e Nitrogênio**	**Referência**
Lactobacillus casei	L(-)	180,0 g/L	Glicose e extrato de levedura	DING e TAN 2006
Lactobacillus delbrueckii	D(-)	162,0 g/L	Melaço e água de maceração de milho	Neste estudo
Lactobacillus lactis		143 g/L	Glicose, extrato de levedura e peptona	YANG et al., 2015
Lactobacillus delbrueckii NCIM 2365	D(-)	135 g/L	Caldo de cana de açúcar e extrato de levedura	KADAM et al. 2006
Lactobacillus delbrueckii	D(-)	120,0 g/L	Caldo de cana de açúcar	CALABIA e TOKIWA, 2007
Lactobacillus delbrueckii ATCC-6949	D(-)	101,0 g/L	Melaço de cana	FRANÇA et al., 2009
Lactobacillus plantarum LMISM6	DL	94,8 g/L	Melaço e água de maceração de milho	COELHO et al. 2011
Lactobacillus delbrueckii NCIMB 8130	D(-)	88,0 g/l	Melaço de beterraba e extrato de levedura	KOTZAMANIDIS et al., 2002
Lactobacillus delbrueckii subsp. *lactis* QU 41	D(-)	20,1 g/L	Glicose e extrato de levedura	TASHIRO et al., 2011
Lactobacillus manihotivorans LMG 18010	L(+)	12,6 g/L	Amido	GUYOT et al., 2000

3.9 Planejamento experimental como ferramenta na otimização de processos

Segundo Hussein (2008), quando um processo é constituído por muitas variáveis e se deseja aprimorá-lo, Delineamento de Experimento é uma metodologia promissora para aperfeiçoar esse tipo processo. O planejamento de experimentos pode ser considerado um

projeto que contém duas divisões, a primeira o delineamento de seleção, e a segunda o delineamento de aprimoramento.

Montgomery (2009) define Delineamento de Experimento como uma ferramenta usada para descobrir relacionamentos de causa e efeito em um determinado processo ou sistema. Ele ainda acrescenta que Delineamento de Experimento é um teste ou uma série de testes, no qual as mudanças intencionais são realizadas nas variáveis de entrada de um processo ou sistema, refletindo em alterações que podem ser identificadas na variável de resposta do processo ou sistema. Dentre os possíveis arranjos a serem utilizados em um delineamento de seleção, o arranjo Plackett-Burman se destaca como o arranjo experimental para identificação de fatores principais (COUTO, 2012).

No ponto de vista de Miller e Sitter (2001, 2005), experimentos de seleção são usados para separar um conjunto de fatores candidatos, e identificar aqueles que realmente exercem uma influência significativa na resposta do processo em melhoria.

Planejamento composto central é uma metodologia de superfície de resposta, ou seja, uma técnica estatística eficaz para otimizar experiências multifatoriais nos processos de formulação (AGARRY et al., 2010). Planejamentos experimentais têm sido frequentemente reportados em otimizações de processos de fermentação lática (NAVEENA et al., 2005; COELHO et al., 2011; LIMA et al., 2010).

3.10 Extração e purificação do ácido lático

As barreiras tecnológicas para a produção de ácido lático com baixo custo estão principalmente no processo de separação e purificação do ácido lático do meio fermentativo. Além disso, a eficiência deste processo é essencial para posterior síntese do PLA, visto que a presença de açúcar, proteínas devem ser mínimas para obtenção de bons resultados na polimerização. Ademais, a recuperação e purificação de ácido láctico a partir de meio de fermentação são muito importantes, pelo fato dos impactos significativos sobre a qualidade do ácido lático, que é a matéria prima para os polímeros. Produtos farmacêuticos ou derivados de alimentos exigem um uma maior pureza do material, no entanto a etapa de purificação é responsável pelo alto custo no produto final (REIMANN, 2006). Assim, para reduzir os custos e os problemas ambientais, muitos estudos sobre a recuperação e purificação de ácido láctico a partir de meios de fermentação têm sido realizados usando diferentes técnicas de separação **(**GONZALEZ et al., 2006; TIMBUNTAM et al., 2008;. MARINOVA et al., 2004).

O método convencional de purificação do ácido lático, por precipitação, é um processo econômico, simples e confiável, porém gera grandes quantidades de sulfato de cálcio, que é

considerado contaminante ambiental (JOGLEKAR et al., 2006). Por esta razão há a procura de métodos alternativos e ecologicamente corretos para a purificação, como por exemplo, métodos envolvendo: troca-iônica, adsorventes em geral, destilação a vácuo, separação por membrana (LI et al., 2008), eletrodiálise, nanofiltração (BOUCHOUX et al., 2006) e osmose reversa (TIMMER et al., 1993).

A purificação de ácido lático a partir de colunas de troca iônica tem se mostrado como uma opção tecnológica para o processo (GONZÁLEZ et al., 2008), uma vez que os equipamentos necessários são relativamente simples, entretanto o uso é recomendado quando a solução de ácido lático possui baixa concentração de sais (QUINTERO et al., 2012). Adsorção em resina de troca iônica é um método prático na indústria, devido a sua economia, facilidade de utilização, redução em consumo de substâncias químicas e menor produção de resíduos. (MOLDES et al., 2003).

O processo de nanofiltração também é uma opção para o processo de separação do ácido lático do meio de fermentação, é um passo de purificação que pode ser realizado antes e/ou depois do processo de troca iônica, pois as membranas de nanofiltração apresentam baixas taxas de rejeição ao ácido lático e alta retenção de mono e dissacarídeos e íons divalentes (Ex. Ca^{+2} e Mg^{+2}) (KANG et al., 2004), além disso é possível obter uma alta taxa de recuperação do ácido lático.

A utilização de carvão ativado, que é um adsorvente, é uma ótima alternativa para a clarificação e para a remoção de quantidades significativas de açúcares e proteínas do meio de fermentação de produção de ácido lático, pois a adsorção de açúcares e proteínas no carvão ativado é maior que a adsorção do ácido lático (CHEN e JU, 1998). Além disso, o processo é de fácil manipulação e apresenta baixo custo. Na maioria dos estudos de purificação de ácido lático, a utilização deste método vem acompanhada de outros processos de purificação (YIN, et al., 1997). Em alguns estudos mencionados em patentes verifica-se que, para obtenção do ácido lático purificado e eliminação de parte ou de todo os íons residuais (sulfato, fosfato), proteínas e açúcares remanescentes (lactose, glicose), são necessários etapas de nanofiltração, carvão ativado e coluna de troca iônica (MANI e HADDEN, 1998).

A eletrodiálise envolve o transporte de íons de uma solução através de uma membrana de troca iônica para outra solução, sobre a influência de uma aplicação elétrica de diferença de potencial (BANASIAK et al., 2007). Esta técnica consiste em um método conveniente para recuperar o ácido lático diretamente do mosto fermentado, com a possibilidade de reciclar o material não extraído para o prosseguimento da fermentação, pois este ainda contém significativas quantidades de elementos necessários para a fermentação (TRINDADE, 2002).

Este sistema de recuperação apresenta maior custo-benefício em comparação com os processos convencionais de *dowsntream*. Consequentemente, o impacto ambiental e os custos operacionais dos processos tradicionais de precipitação podem ser reduzidos (WANG, 2012). Alguns estudos sobre a utilização de eletrodiálise na recuperação de ácido láctico a partir de lactato de sódio foram relatados (KIM e MOON, 2001; HABOVA et al., 2004).

A necessidade de se estudar procedimento para purificação do ácido lático envolve operações unitárias, sendo que as etapas críticas são avaliadas em relação ao rendimento na recuperação do ácido lático, remoção de contaminantes como proteínas, açúcares e cor.

4 Metodologia e Resultados

Os materiais e métodos específicos utilizados, assim como os resultados estão descritos nos próximos capítulos correspondentes aos artigos científicos.

Capítulo I- Artigo publicado na revista *Annals of Microbiology* (versão publicada em anexo)

High D(-) lactic acid levels production by *Sporolactobacillus nakayamae* and an efficient purification

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Abstract

This study is the first to report the efficient production of D(-) lactic acid by a strain of *Sporolactobacillus nakayamae* utilizing inexpensive peanut flour as a nitrogen source and commercial sucrose as a carbon source. A D(-) lactic acid concentration of 112.93 g/L was obtained by fermentation using 110.00 g/L of commercial sucrose, 150.40 g/L of peanut flour and 0.16 mL/L of Tween 80. The optimal medium composition was determined by using experimental design as a Plackett-Burman, fractional factorial designs and response surface methodology. The purification and recovery of D(-) lactic acid from the fermentation medium were performed using two purification techniques (filtration with activated carbon, followed by celite and cation resin exchange). This approach showed a high efficiency in protein and sugar removal as well in D(-) lactic acid recovery from the fermentation medium. Thus, the results indicate that *S. nakayamae* is a promising new D(-) lactic acid producer.

Keywords: D(-) Lactic acid; isomers; *Sporolactobacillus nakayamae*; purification; submerged fermentation.

Introduction

The production of lactic acid by microorganisms has gained a prime position in industry because it is cost effective and eco-friendly. Lactic acid is a versatile chemical with a wide variety of applications across a range of industries (John et al. 2009; Djukic´-Vukovic´ et al. 2012). Lactic acid can be produced by fermentation from different raw materials and through various technological means; additionally, it has versatile properties as a precursor for various chemicals and materials (Kim et al. 2006; Zhang et al. 2007; Hetényi et al. 2008; Dumbrepatil et al. 2008).

Approximately 40% of produced lactic acid is used in manufacturing polylactic acid (Bozell and Petersen 2010), which has recently been studied with great interest as a biodegradable polymer that can be used in the production of food packaging, plastic utensils, and plastic sheeting used for parts of computers, thereby replacing products made from petroleum (Ohara 2003; Coelho et al. 2011). L(+) Lactic acid is used in the food industry to preserve human foodstuffs and is also utilized in the pharmaceutical and cosmetic industries (Li et al. 2004; Naveena et al. 2005; Wee et al. 2006).

Some microorganisms can produce optically pure L(+) or D(-) lactic acid isomers via fermentation, while racemic DL-lactic acid is produced via chemical synthesis (Hofvendahl and Hahn-Hägerdal 2000; Lima et al. 2010). In the literature, there are more studies related with L (+) lactic acid than D(-)- lactic acid production, additionally, the production of D(-) lactic acid is lower than that L(+) lactic acid (Wang et al. 2011).

Designs experiments have been frequently used for optimizing media to improve lactic acid production (Chauhan et al. 2006; Mel et al. 2008). Methods such as factorial design and response surface methodology provide powerful and efficient ways to optimize cultivation and procedures using a reduced number of experiments (Mandenius and Brundin 2008).

The quality of the lactic acid from fermentation media is closely related to recovery and purification methodologies (González et al. 2006). Typically, lactic acid for use in polymers, pharmaceuticals, or food derivatives requires further purification steps; however, the inclusion of these steps is responsible for the high cost of lactic acid obtained by fermentation (Timbuntam et al. 2008). Thus, to reduce costs and solve environmental problems, many studies of the recovery and purification of lactic acid from fermentation media have been conducted using different separation techniques (Marinova et al. 2004; González et al. 2006; Timbuntam et al. 2008).

The present study reports the highly efficient production of D(-) lactic acid by *S. nakayamae* using peanut flour as a nitrogen source to reduce the production cost. Fermentation conditions were optimized through

experimental design. Additionally, a new methodology for the recovery and purification of D(-) lactic acid from the fermentation medium by filtration through activated carbon and Celite, followed by cation exchange chromatography was present.

Materials and Methods

Bacteria strain and culture conditions

No specific permissions were required for these locations because this microorganism was collected within the university. *S. nakayamae*, a homofermentative lactic acid bacterium which produces D(−) lactic acid with high optical purity, was used in this study. This microorganism was isolated from the rhizosphere of *Desmodium adscendens* collected in the state of São Paulo (Brazil). The strain was stored in GYP medium with 20% (by volume) glycerol at -80 °C. GYP medium is composed of 2% glucose, 1% yeast extract, 1% peptone, 1% sodium acetate and 0.5% (v/v) salt solution, which was comprised of 4% $MgSO_4 \cdot 7H_2O$, 0.16% $MnSO_4.4H_2O$, 0.2% $FeSO_4.7H_2O$ and 0.2 % NaCl; pH 7.0.

Inoculum and shake flask fermentation

Inoculum was prepared from activated culture grown on GYP medium incubated at 35±1 °C under stationary conditions until the OD_{600} reached 2.5 (approximately 18-20 h). Experiments were performed in 125 mL flasks containing 18 mL of GYP medium at pH 7.0 and inoculated with 2 mL of the seed culture (10% inoculum). To avoid a pH decrease because of lactic acid production, 50 g/L of $CaCO_3$ was added to each flask. The temperature was kept at 35 ºC. The cultures were incubated at different conditions, as described below. All experiments were carried out in duplicate to verify their reproducibility, and the results are expressed as the mean values. The fermentation samples were centrifuged at 7000 x g for 15 minutes. The resulting supernatant was filtered through a 0.22 μm membrane and was then used to determine the concentration of residual sugar and D(-) lactic acid .

Lactic acid production on different carbon and nitrogen sources

Lactic acid production was studied with different substrates as carbon and nitrogen sources based in GYP medium, incubation for 48 hours at 35 °C under shaker conditions at 150 rpm.

After the sources that induced the highest lactic acid level were selected, several concentrations of the carbon and nitrogen sources were evaluated using the experimental designs described below.

Experimental designs

Screening of significant nutritional components of lactic acid production using the Plackett-Burman Experimental Design (PBED)

The PBED was employed for screening and identifying the significance of eight parameters for D(-) lactic acid production. The GYP medium was modified to evaluate the influence of different nutritional components on lactic acid production. Each factor was tested at two levels (coded): high level (+ 1) and a low level (-1) (Table 1), and four central points were screened by running 16 experiments. The factors that were significant at the 5% level ($P < 0.05$) from the regression analysis were considered to have a high impact on lactic acid production.

Table 1 Variables and their levels in lactic acid production under a PBED

Fractional factorial designs

Five variables were studied in a 2^{5-1} FFD to estimate their effect on lactic acid production. A total of 19 experiments were carried out, with 16 experiments related to the design structure and 3 repetitions at the central point. The two extreme levels are denoted by minus one (lower level) and plus one (higher level), as shown in Table 2.

Table 2 Design matrix for the fractional 2^{5-1} factorial experiments, used to study the influence of 5 factors on *S. nakayamae* D(-) lactic acid production

A new approach of GYP salts was evaluated, and it was added to the solution at certain concentrations. Once peanut flour was established as a nitrogen source, the influence of B vitamins on the production of lactic acid was examined. The fermentation was carried out under shaker conditions (100 rpm) at 35 ºC for 48 hours. After incubation, the cultures were processed and quantitatively assayed for lactic acid and residual sugar production.

Response Surface Methodology (RSM)

The RSM was applied to evaluate the interaction of variables and then used to find the optimum concentration of the medium components that greatly affect D(-) lactic acid production (Table 3). To study the influence of commercial sucrose, peanut flour, and Tween 80 on the production of lactic acid by *S. nakayamae*, an RSM was used, with 4 replicates at the center, totaling 18 experiments.

Table 3 Variables and their levels of lactic acid production under an RSM

The flasks were incubated for 72 hours at 35 °C under shaker conditions at 100 rpm. Lactic acid and residual sugar were quantified.

Experimental validation

To validate the optimum values selected by the response surface analysis, two conditions were tested. The concentrations utilized were: 110.00 g/L of commercial sucrose, 150.40 of peanut flour and two different concentration of Tween 80 being 1.00 mL/L and 0.16 mL/L.

The fermentation was carried out under shaker conditions (100 rpm) at 35 ºC for 72 hours. Each condition was tested five times, and the results are expressed as the mean values.

Purification and recovery of D(-) lactic acid

The fermentation medium was centrifuged at 7000 x *g* at 20 ˚C for 10 min, and the pH was adjusted to 5.0. Then, 60 mL of the medium (88.27 g/L D(-) lactic acid and 13.50 g/L of sugar) was vacuum filtered through a sintered plate funnel (3.50 cm diameter and 5.00 cm high) containing 1 cm of activated carbon and 1 cm of Celite. The filtrate was filtered through activated carbon and Celite twice, then passed through a cation exchange column (Ambertile IRA 120 hydrogen forms), and filtered through a 0.22 μm membrane. Then, the final solution was used to determine the lactic acid, sugar and protein concentrations. The total protein concentration was determined by the Lowry method (Peterson 1977).

The lactic acid recovery yield was calculated as follow: lactic acid recovery yield (%) = (final mass (g) / initial mass (g)) x 100.

The sugar removal was calculated using this formula: sugar removal (%) = ((initial mass (g) - final mass (g))/ initial mass (g)) x 100.

Analytical techniques

The concentrations of sucrose, lactose and lactic acid were determined by high performance liquid chromatography (HPLC) using a column Rezex ROA 300 mm x 7.8m m (Phenomenex, USA) and a differential refracting index detector (Shimadzu Ultra Fast Liquid Chromatograph, Japan). The mobile phase (0.005 M H_2SO_4) was fed at a flow rate of 0.6 mL/min, and the temperature was kept 65 °C.

In the end of the optimization process of D(-) lactic acid production, the optical purity of lactic acid produced was determined by HPLC using a Chirex 3126 phenomenex (150×4.6 mm) column with 1 mM of CuSO4 as the mobile phase at 1 mL/min (26 °C).

Statistical analysis

The experimental design and the results analysis in all experimental designs were determined using STATISTICA 7 (StatSoft, Tulsa, USA).

Results and discussion

Influence of carbon sources

D(-) Lactic acid production by *S. nakayamae* under the influence of four inexpensive carbon sources was studied (Table 4).

Table 4 Influence of carbon sources on *S. nakayamae* lactic acid production, productivity and yield

The carbon source that induced the highest lactic acid production was commercial sucrose, producing 59.50 g/L of lactic acid. *S. nakayamae* was also able to produce lactic acid in the presence of sugarcane juice, yielding 53.80 g/L of lactic acid. In the presence of whey and molasses, low levels of lactic acid were produced compared to those of the cultures grown with commercial sucrose and sugarcane juice. It may be that growing the cultures with molasses produced low levels of lactic acid because of the heavy metals (iron, zinc, copper, manganese) found in this substrate, which may inhibit cell growth and inactivate the enzymes involved in final product conversion. However, in previous studies, it was shown that L(+) lactic acid production by *Enterococcus faecalis* from molasses reached a maximum concentration of 134.9 g/L, with a maximum productivity of 4.3 g/Lh (Wee et al, 2006). In the presence of whey, the low level of D(-) lactic acid production should be caused by inability of *S. nakayamae* metabolize the lactose, that is the sugar present in this residue, as reported before by

Sanders et al., 2003, when referring to the other species of the genus *Sporolactobacillus.* In contrast, in a previous study, it was reported that 52.5 g/L of lactic acid was produced from whey by *Lactobacillus helveticus* (Ghaly et al. 2004).

Among the sources tested, commercial sucrose was selected. Although sugarcane juice was also capable of inducing lactic acid production, this substrate is less interesting in regard to further purification.

Influence of nitrogen sources

Different sources of nitrogen were also studied: corn-steep liquor, Proflo, peanut flour, soybean flour and urea. The effects of each of these nitrogen sources on lactic acid production are shown in Table 5:

Table 5 Influence of nitrogen sources on *S. nakayamae* lactic acid production, productivity and yield

The nitrogen source that induced the highest lactic acid production was peanut flour, corresponding to 57.00 g/L of lactic acid. Moreover, Proflo, induced the production of 52.2 g/L of D(-) lactic acid, and a yield of 0.70 g of lactic acid/g of Proflo. Thus, it was the second most efficient nitrogen source in the production of lactic acid by *S. nakayamae*, this source presents some vitamins on composition, possibly these vitamins have a role on this fermentation. Peanut flour and soybean flour have a several mineral in their composition (Calcium, Iron, Magnesium, Manganese and Sodium), which can act as enzyme cofactors. Similar to this study, it has been previously shown that *Bacillus* sp. and *Sporolactobacillus* sp. CASD produces high levels of L(+) and D(-) lactic acid respectively, using peanut flour as its sole nitrogen source (Meng et al. 2012; Wang et al. 2011).

Moreover, in another previous study, it was reported that *Lactobacillus* sp. produced 41.42 g/L of D(-) lactic acid using corn step liquor and yeast autolysate (Lima et al. 2009). In the present study, when only corn step liquor was used as a nitrogen source, the lactic acid production was not as efficient, yielding 13.26 g/L of D(-) lactic acid . Furthermore, it left a high concentration of residual sugar (70.00 g/L).

Because the nitrogen source accounts for approximately 38% of the total process cost during lactic acid production (Altaf et al. 2007), the use of wastes and inexpensive nitrogen sources in fermentation medium would reduce these costs.

Experimental designs

Plackett-Burman Experimental Design

The PBED is an efficient screening method for identifying the active factors of an experiment using as few experimental runs as possible.

The matrix (coded values) of the 16 experiments with eight variables and the respective results were shown in Table 6. After 48 hours of fermentations, the maximum level of lactic acid production was 58.90 g/L (run 7).

Table 6. PBED with coded values and experimental results for lactic acid production, productivity, yield and residual sugar concentration

The Pareto graph implied that the variables $MgSO_4$, peanut flour, Tween 80 and commercial sucrose influenced the fermentation process significantly at a 95% confidence level. $MgSO_4$ and commercial sucrose had negative coefficients, while peanut flour and Tween 80 showed positive coefficients (Figure 1a).
Kitouni and Oulmi (2013) using PBED reported that the use of manganese, magnesium and iron sulfates as sources of oligoelements appears to have no significant effect on the lactic acid production by *Lactobacillus bulgaricus* attc 11842 grown on whey.

Once the peanut flour presents high lipid concentration in the composition (51.77 g/100 g), the surfactant property of Tween 80 can interact with lipids, improving the solubility of the nutrients found in peanut flour. Tween 80 may assist in nutrient absorption by microorganisms, as well as product liberation by facilitating transport via the cell membrane of the microorganism. Conversely, Coelho et al., 2011 reported that Tween 80 had a significant positive effect on lactic acid production by *Lactobacillus plantarum* LMISM6.

The negative effect of commercial sucrose is probably related to the high concentration used, which could inhibit the lactic acid production. Therefore, the components selected for the next experiment were peanut flour, commercial sucrose, and Tween 80.

Several studies reported the use of PBED successfully employed to screen the significance components in culture media, in order to improve the lactic acid production (Wang et al., 2015; Qin et al., 2009; Chauhan et al., 2007). Gowdhaman et al., (2012) using PBED was able to select three variables namely substrate concentration, moisture content, and yeast concentration that have statistically significant effect on lactic acid yield by *Lactobacillus plantarum* MTCC 6161 in solid state fermentation. Chauhan et al., 2006 studying optimization of

lactic acid production using date juice reported that among the fifteen variables (date juice, peptone, beef extract, yeast extract, K2HPO4, KH2PO4, MgSO4·7H2O, MnSO4·H2O, sodium acetate, sodium sulfate, tri-sodium citrate, sodium succinate, tween-80, FeSO4 and NaCl), significant components were screened out using PBED . This experiment suggested four components, date juice, peptone, K2HPO4, and sodium acetate, as having significance effect on lactic acid production by *Lactobacillus* sp. KCP01.

Fractional factorial designs (FFD)

In this study, a 2^{5-2} FFD was used to determine the significant factors of five media components. The 19 runs and the results of the response FFD are presented in Table 7.

Table 7 Fractional factorial design with coded and experimental results for lactic acid production, productivity, yield and residual sugar concentration

The highest lactic acid concentration was identified on run 8 (52.92 g/L). This run contained the highest concentrations of carbon and nitrogen sources and contained no salts or vitamins, however the level of residual sugar found in this experiment was high (60.04 g/L).

After 48 hours of fermentation, the peanut flour had a positive and significant effect on D(-) lactic acid production (Figure 1b):

Fig 1 Pareto graph for D(-) lactic acid production. a) PBED; b) FFD

The commercial sucrose and salts, despite presented as not significant influence, had negative effects on lactic acid production. This negative effect may be associated with the amount of carbohydrates and mineral salts have already existing in peanut flour, causing high concentration of these components on the culture media. As previously mentioned, the negative effect of commercial sucrose is related to product inhibition mediated by high substrate concentration. Different results were reported by Vasala et al., (2005) where they showed that *Lactobacillus salivarius* ssp. it is an ideal organism for lactic acid fermentation in high-salt high lactose conditions. Additionally, the vitamins and Tween 80 did not significantly influence lactic acid production, nevertheless, it was verified that when the highest concentration of Tween 80 was used, there was higher lactic

acid production. However, previous studies reported a positive influence of B vitamins on lactic acid production (Yoo et al. 1997; Altaf et al. 2007).

Considering these results, Tween 80, commercial sucrose and peanut flour were selected to use in the RSM.

Response Surface Methodology (RSM)

Three factors, commercial sucrose, peanut flour and Tween 80, were distributed in three levels, two axial points and four central points were evaluated. The responses measured were D(-) lactic acid concentration, productivity, yield and residual sugar after 72 h of fermentation (Table 8). Among the concentration of nutrients evaluated, 130 g/L of commercial sucrose, 130 g/L of peanut flour and 1.5 mL/L of Tween 80 (run 8) achieved the highest D(-)lactic acid production (101.99 g/L) and productivity (1.42 g/L h), although there was a high concentration of residual sugar (26.13 g/L).

Table 8 RSM design with coded values and experimental results for lactic acid production, productivity, yield and residual sugar concentration

The results obtained were fitted to multiple regression analysis to yield the following regression equation:

$$Y = 91.62 + 5.22X_1 + 2.75X_2 + 1.12X_3 - 2.71X_{1^2} + 2.64X_1X_2 + 1.30X_1X_3 + 0.37X_{2^2} - 2.08X_2X_3 + 0{,}02X_{3^2}$$

in which Y is the predicted response (D(-) lactic acid production) and X1, X2, and X3 are the coded values of the test variables for commercial sucrose, peanut flour and Tween 80, respectively.

An ANOVA of the quadratic regression model demonstrated that the model is significant, as determined by Fisher's test (Fcalc (3.81) > Ft (3.39)). It was also determined that the model has a very low probability value ((Pmodel>F) =0.03). The fit of the model was checked by the coefficient of determination (R^2) and multiple correlation coefficient (R). The R^2 value (0.81) for the regression equation indicated that the sample variation for lactic acid of 81% was attributed to the independent variables. In this case, the value of the coefficient of determination indicated that only 19% of the total variations were not explained by the model. The high R value (0.90) demonstrated a strong agreement between observed and predicted values. Among the model terms, X1 was significant with a probability of 95%.

The response for the regression equation was plotted in Figs. 2-4. The following graphs show the interaction of the variables and the optimum levels of each variable for D(-) lactic acid production.

Fig 2 Response surface for lactic acid production by *S. nakayamae* showing the interaction between peanut flour and commercial sucrose

Fig 3 Response surface for lactic acid production by *S. nakayamae* showing the interaction between Tween 80 and commercial sucrose

Fig 4 Response surface for lactic acid production by *S. nakayamae* showing the interaction between Tween 80 and peanut flour

Based upon the response surfaces and the values presented in Table 8, run 12 was considered the most appropriate combination for D(-) lactic acid production. This run consisted of 110 g/L of commercial sucrose, 150.40 g/L of peanut flour, and 1 mL/L of Tween 80, producing 99.99 g/L of D(-) lactic acid, with a 1.39 g/Lh rate of productivity, and a low concentration of residual sugar (10.24 g/L).

When the highest levels of commercial sucrose and peanut flour were used, it was verified that this condition produced high levels of lactic acid production; however, the amount of residual sugar produced was substantial. The same results were verified on commercial sucrose and Tween 80 interaction. The response surface between peanut flour and Tween 80 showed that intermediate concentrations of this components did not produce a satisfactory amount of D(-) lactic acid . Additionally, it was found that low Tween 80 concentrations combined with high peanut flour concentrations showed higher levels of D(-) lactic acid production.

Previous studies have reported that RSM is an efficient method for the determination of optimized culture conditions for the production of lactic acid (Lima et al. 2011; Coelho et al. 2011). Chaisu et al. (2014) reported, using RSM, the best percentage of inoculum (8.02%) and carbon source (3.82% of molasses) to lactic acid production (38.33%) by *Lactobacillus casei* M-15 within 24 hr at 37 °C. Bustos et al., demonstrated the optimization of culture media applying RSM using *Lactobacillus coryniformis.* The models predicted a maximum D(-) lactic acid concentration (58.9 g/L) at 96 h using 5 g Corn Steep Liquor/L, 3.6 g Yiest Extract/L and 10 g Peptone/L. In turn, Lima et al., 2010 through RSM verify that the best results for lactic acid production

(52.37 g/L) were obtained with 59.64 g/L of lactose, 14.55 g/L of Corn Steep Liquor and 5.65 g/L of (NH4)2SO4, at 39.6 °C by *Lactobacillus* sp. LMI8.

Experimental validation

A low concentration of Tween 80 (0.16 mL/L) combined with 110 g/L of commercial sucrose and 150.40 g/L of peanut flour, induced a higher D(-) lactic acid production (112.93 g/L), productivity (1.57 g/L.h) and yield (0.98 g/g) with a reduced residual sucrose concentration (9.6 g/L). Compared with the condition were it was utilized a higher concentration of Tween 80 (1.00 mL/L), low levels of D(-) lactic acid (104,38 g/L), productivity (1,45 g/L.h) and yield (0,96 g/g) were presented.

In order to verify the optical purity of lactic acid produced, the optimizated culture media was analyzed by HPLC using a chiral column. *S. nakayamae* produced 98.75% of D(-) lactic acid and 1.24% of L(+) lactic acid, thus it is considered homofermentative microorganism.

Purification and recovery of D(-) lactic acid

The centrifuged fermentation medium at pH 5.0 was submitted to the purification process, which involved two steps: 1) double filtration on activated carbon and Celite, and 2) the transformation of calcium lactate into lactic acid via cation resin exchange. The purification and recovery assays were performed in duplicate, and the results are expressed as the mean values (Table 9).

Table 9 Purification parameters of the fermentation media

D(-) lactic acid was efficiently recovered from fermentation medium (79 %). Additionally, protein and sugar were efficiently removed (100 % and 98 %, respectively). Previous studies have been reported similar recovery yields using other purification techniques, such as solvent extraction, multiple-pass distillation, and micellar-enhanced ultrafiltration (Chen et al. 2012; Geanta et al, 2013). Furthermore, it was verified that there was a low ratio of sugar to lactic acid (0.36 %) which is essential to polymer synthesis for avoiding caramelization.

Conclusions

In this study, it was found that *S. nakayamae* is able to produce high levels of D(-) lactic acid using peanut flour and commercial sucrose as substrates. Experimental design methodology effectively determined the significant nutrients and optimum concentration of medium components. The purification methodology works with inexpensive and reusable reagents. In addition, the high performance of this method of D(-) lactic acid production utilizing cheap materials will be beneficial for bioplastic technologies. However, to be applied to a large scale in the lactic acid industries, more well-established research efforts need to conducted.

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Figure legends

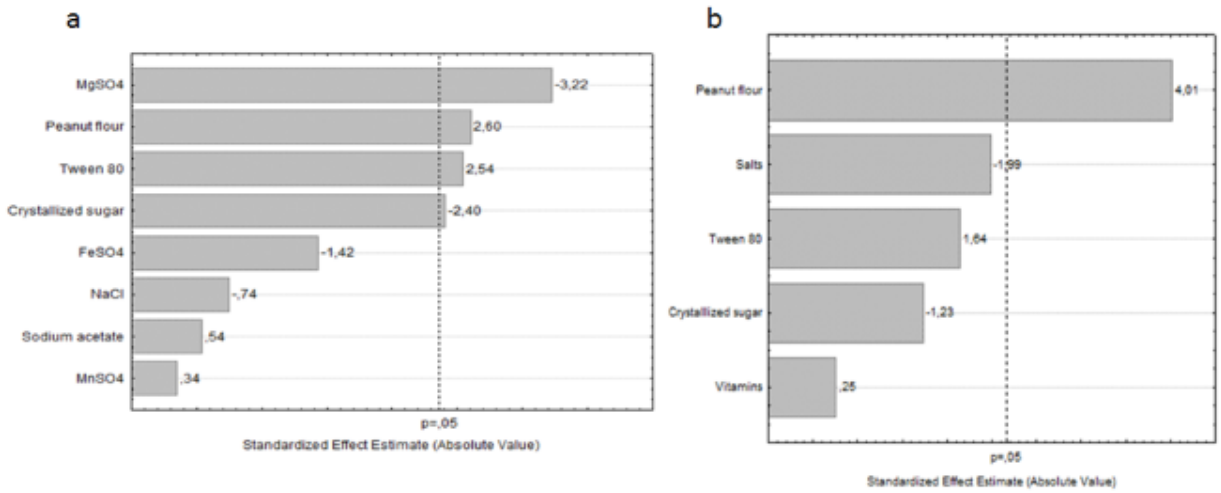
Fig 1 Pareto graph for D(-) lactic acid production. a) PBED; b) FFD

Fig 2 Response surface for lactic acid production by *S. nakayamae* showing the interaction between peanut flour and commercial sucrose

Fig 3 Response surface for lactic acid production by *S. nakayamae* showing the interaction between Tween 80 and commercial sucrose

Fig 4 Response surface for lactic acid production by *S. nakayamae* showing the interaction between Tween 80 and peanut flour

Fig 1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Tween 80
Crystallized sugar
FeSO4
NaCl
Sodium acetate
MnSO4
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2,60
2,54
-2,40
-1,42
-,74
,54
,34
p=,05
Standardized Effect Estimate (Absolute Value)
b
Peanut flour
Salts
Tween 80
Crystallized sugar
Vitamins
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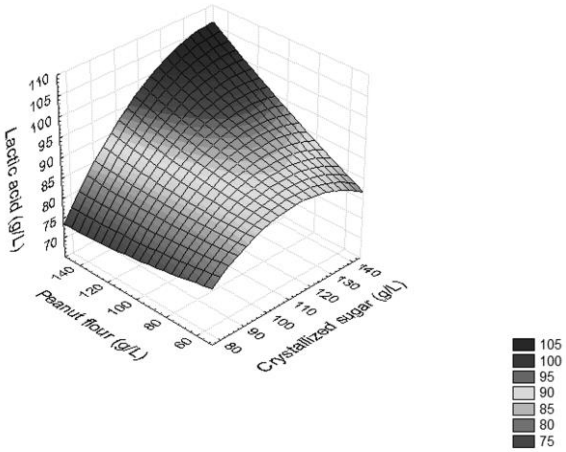
1,64
-1,23
,25
p=,05
Standardized Effect Estimate (Absolute Value)

Fig 2


Lactic acid (g/L)
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105
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Peanut flour (g/L)
140
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60
Crystallized sugar (g/L)
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130
140
105
100
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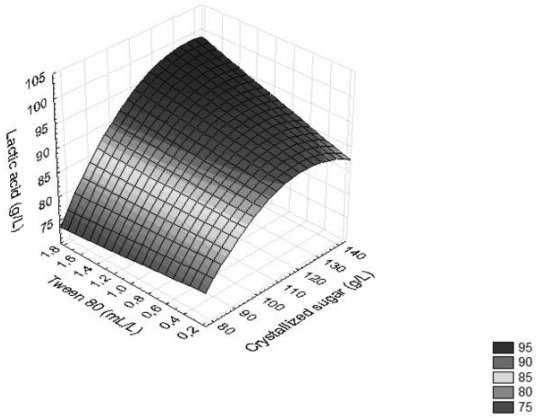
Fig 3


Lactic acid (g/L)
105
100
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90
85
80
75
Tween 80 (mL/L)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Crystallized sugar (g/L)
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130
140
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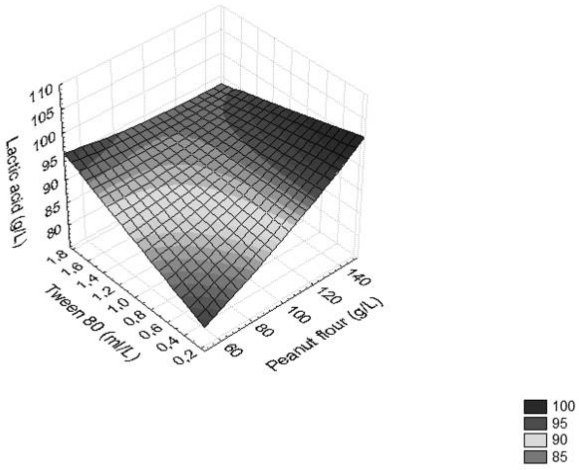
90
85
80
75

Fig 4


Lactic acid (g/L)
110
105
100
95
90
85
80
Tween 80 (ml/L)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Peanut flour (g/L)
60
80
100
120
140
100
95
90
85

Tables

Table 1. Variables and their levels in lactic acid production under a PBED

Variable	Code	Levels	
	Low (-1)	0	High (+1)
Tween80 (mL/L)	X_1	0	0.5	1
$MnSO_4$ (g/L)	X_2	0	0.05	0.1
$FeSO_4$ (g/L)	X_3	0	0.05	0.1
NaCl (g/L)	X_4	0	0.05	0.1
$MgSO_4$ (g/L)	X_5	0	1	2
Peanut flour (g/L)	X_6	40	60	80
Sodium acetate (g/L)	X_7	0	5	10
Commercial sucrose (g/L)	X_8	80	115	150

Samples were incubated at a temperature of 35 ± 1ºC for 48 hours at 100 rpm. A Fractionated Factorial Design (FFD) was performed according the variables that significantly influenced the production of lactic acid.

Table 2. Design matrix for the fractional 25-1 factorial experiments, used to study the influence of 5 factors on *S. nakayamae* D(-) lactic acid production

Variable		Code	Levels	
		Low (-1)	0	High (+1)
Commercial sucrose (g/L)	X_1	80	100	120
Peanut flour (g/L)	X_2	60	80	100
Tween 80 (mL/L)	X_3	0.5	1	1.5
GYP Salts solution (g/L)	X_4	0	2.5	5
Vitamins (mL/L)	X_5	0	5	10

Tabela 3. Variables and their levels of lactic acid production under an RSM

Variable		Levels				
		- 1.68	-1	0	+1	+1.68
Commercial sucrose (g/L)	X_1	76.40	90	110	130	143.60
Peanut Flour (g/L)	X_2	49.60	70	100	130	150.40
Tween 80 (mL/L)	X_3	0.16	0.5	1.00	1.5	1.84

Table 4. Influence of carbon sources on *S. nakayamae* lactic acid production, productivity and yield

Carbon sources	Carbohydrates	D(-) Lactic acid Production (g/L)	Productivity (g/Lh)	Yield Yp/s (g/g)	Residual sugar (g/L)
Molasses	46,9 (g/100mL)[a]	1.06	0.02	0.12	91.40
Commercial sucrose	100 (g/100g)[a]	59.50	1.23	0.69	14.80
Sugarcane juice	19.95 (g/100mL)[a]	53.80	1,12	0.66	19.20
Whey	76.59 (g/100g)[b]	1.26	0.02	0.11	88.57

Culture conditions: GYP medium with 100 g/L of carbon source, 5% of $CaCO_3$, at 35 °C under shaker conditions at 150 rpm, for 48 h, with an initial pH of 7.0. Type of carbohydrate: a)sucrose; b) lactose

Table 5. Influence of nitrogen sources on *S. nakayamae* lactic acid production, productivity and yield

Nitrogen sources	N %	D(-) Lactic acid Production (g/L)	Productivity (g/Lh)	Yield Yp/s (g/g)	Residual sugar (g/L)
Proflo	11.15	52.20	1.09	0.71	26.43
Corn step liquor	4,69	13.26	0.28	0.44	70.00
Urea	46.62	38.40	0.80	0.78	51.00
Soybean flour	6.40	41.28	0.86	0.60	30.81
Peanut flour	7.63	57.00	1.19	0.72	21.2

Culture conditions: GYP medium with 100 g/L of commercial sucrose, 5% of $CaCO_3$, at 35 °C under shaker conditions at 150 rpm, for 48 h, at an initial of pH 7.0. The nitrogen content of all media was 15.25% ,

Table 6. PBED with coded values and experimental results for lactic acid production, productivity, yield and residual sugar concentration

Run	Independent variables								Lactic acid	Productivity	Yield Y p/s	Residual Sugar
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	g/L	g/L/h	g/g	g/L
1	1	-1	1	-1	-1	-1	1	1	52.44	1.09	0.80	84.35
2	1	1	-1	1	-1	-1	-1	1	44.40	0.92	0.69	86.02
3	-1	1	1	-1	1	-1	-1	-1	30.43	0.63	0.99	49.15
4	1	-1	1	1	-1	1	-1	-1	55.85	1.16	0.98	22.92
5	1	1	-1	1	1	-1	1	-1	51.05	1.06	0.97	27.63
6	1	1	1	-1	1	1	-1	1	45.78	0.95	0.78	91.09
7	-1	1	1	1	-1	1	1	-1	58.90	1.22	0.99	23.75
8	-1	-1	1	1	1	-1	1	1	15.64	0.32	0.76	133.72
9	-1	-1	-1	1	1	1	-1	1	40.96	0.85	0.77	96.73
10	1	-1	-1	-1	1	1	1	-1	54.73	1.14	0.98	24.42
11	-1	1	-1	-1	-1	1	1	1	48.70	1.01	0.40	98.93
12	-1	-1	-1	-1	-1	-1	-1	-1	51.73	1.08	0.95	25.42
13	0	0	0	0	0	0	0	0	51.19	1.07	0.96	61.61
14	0	0	0	0	0	0	0	0	46.38	0.97	0.76	53.70
15	0	0	0	0	0	0	0	0	50.25	1.05	0.78	50.60
16	0	0	0	0	0	0	0	0	51.59	1.07	0.94	59.97

X_1= Tween80. X_2= $MnSO_4$. X_3= $FeSO_4$. X_4=NaCl. X_5= $MgSO_4$. X_6= Peanut flour. X_7= Sodium acetate. X_8= Commercial sucrose. Culture conditions: 35ºC, initial pH of 7.0 neutralized by 5% $CaCO_3$, 100 rpm of agitation, 10% inoculum. Fermentation was carried out for 48 hours.

Table 7. Fractional factorial design with coded and experimental results for lactic acid production, productivity, yield and residual sugar concentration

Run	Independent variables					Lactic acid	Productivity	Yield Yp/s	Residual sugar
	X_1	X_2	X_3	X_4	X_5	g/L	g/Lh	g/g	g/L
1	-1	-1	-1	-1	1	48.49	1.01	1.20	39.70
2	1	-1	-1	-1	-1	41.76	0.87	0.48	33.15
3	-1	1	-1	-1	-1	47.82	1.00	0.67	8.70
4	1	1	-1	-1	1	46.46	0.97	0.70	53.74
5	-1	-1	1	-1	-1	46.24	0.96	0.62	5.35
6	1	-1	1	-1	1	44.35	0.92	0.85	67.86
7	-1	1	1	-1	1	49.02	1.02	0.97	29.21
8	1	1	1	-1	-1	51.92	1.08	0.87	60.04
9	-1	-1	-1	1	-1	42.79	0.89	0.99	36.85
10	1	-1	-1	1	1	43.05	0.90	0.80	66.22
11	-1	1	-1	1	1	46.87	0.98	1.04	35.08
12	1	1	-1	1	-1	44.01	0.92	0.83	66.79
13	-1	-1	1	1	1	42.16	0.88	1.01	38.08
14	1	-1	1	1	-1	42.79	0.89	0.80	66.63
15	-1	1	1	1	-1	49.59	1.03	0.98	29.53
16	1	1	1	1	1	48.59	1.01	0.98	70.22
17	0	0	0	0	0	43.15	0.90	0.85	49.41
18	0	0	0	0	0	44.205	0.92	0.89	50.13
19	0	0	0	0	0	44.465	0.93	0.86	48.52

X_1= Commercial sucrose X_2= Peanut flour. X_3= Tween 80. X_4= Salt solution. X_5= Vitamins. Culture conditions: 35ºC, initial pH 7.0 neutralized by 5% $CaCO_3$, 100 rpm of agitation, 10% inoculum. Fermentation was carried out for 48 hours.

Tabela 8. RSM design with coded values and experimental results for lactic acid production, productivity, yield and residual sugar concentration

Run	Independent variables			Lactic acid	Productivity	Yield Y p/s	Residual sugar
	X_1	X_2	X_3	g/L	g/Lh	g/g	g/L
1	-1	-1	-1	78.10	1.08	0.88	15.20
2	1	-1	-1	85.48	1.19	0.89	54.97
3	-1	1	-1	81.67	1.13	0.79	9.95
4	1	1	-1	99.46	1.38	0.85	22.85
5	-1	-1	1	83.77	1.16	0.92	9.89
6	1	-1	1	96.18	1.34	0.86	28.32
7	-1	1	1	78.81	1.09	0.91	6.80
8	1	1	1	101.99	1.42	0.91	26.13
9	-1,68	0	0	82.40	1.14	0.95	5.39
10	1,68	0	0	88.68	1.23	0.79	41.50
11	0	-1,68	0	88.53	1.23	0.91	23.80
12	0	1,68	0	99.99	1.39	0.86	10.24
13	0	0	-1,68	93.47	1.30	0.84	16.24
14	0	0	1,68	93.11	1.29	0.84	17.92
15	0	0	0	93.53	1.30	0.88	20.88
16	0	0	0	91.60	1.27	0.84	17.80
17	0	0	0	88.80	1.23	0.85	22.66
18	0	0	0	92.02	1.28	0.85	19.12

X1= Commercial sucrose, X2 = Peanut flour, X3 Tween 80. Culture conditions: 35ºC, Initial pH 7.0 neutralized by 5% $CaCO_3$, 100 rpm of agitation, 10% inoculum. Fermentation was carried out for 72 hours.

Table 9. Purification parameters of the fermentation media

Parameters	Lactic acid	Sugar	Protein
[Initial solution] (g/L)*	88.27	13.50	13.54
Initial mass (g)	5.296	0.810	0.812
[Final solution] (g/L)**	16.85	0.06	0.00
Final mass (g)	4.212	0.015	0.000
Index	Recovery Yield	sugar removal	protein removal
	79 %	98 %	100%

* Initial volume 60 mL; ** Final volume 250 mL

Capítulo II- Artigo submetido à revista *Biochemical Enginnering Journal*

Environmentally friendly production of D (-) lactic acid by *Sporolactobacillus nakayamae*: Investigation of fermentation parameters and fed-batch strategies

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Abstract

Due to the wide range of applications of lactic acid, the interest of this production has increased. Parameters affecting the fermentative D(-) lactic acid production are investigated: neutralizing agents, pH, temperature, inoculum percentage, agitation and components medium concentration. Experimental design was applied to determine the best concentration of media components. Also the fermentation using different feeding strategies was studied. High production of D(-) lactic acid (122.41g/l) and productivity (3.65 g/L.h) was efficiently achieved on multi-pulse fed-batch in 54 h by *Sporolactobacillus nakayamae* using initial media containing 35 g/L yeast extract (byproduct of Alcohol production), 60 g/L crystallized sugar and 7.5 mL/L salts solution. The fermentation process was carried at 35 ºC and pH 6.0 controlled by NaOH with 20% volume of inoculum and 125 rpm agitation. The production of a high optically pure concentration D(-) lactic acid combined with a friendly NaOH based process, demonstrated that *S. nakayamae* is a promising strain for D(-)lactic acid production.

Keywords: D(-) lactic acid, pH controller, *Sporolactobacillus nakayamae*, fermentation, fed-batch fermentation

1. Introduction

Lactic acid production have received more attention due to its high potential for biotechnological applications in a wide range of fields, as well because of the increasing need for new biomaterials such as biodegradable and biocompatible polylactic products [1-3].

There are two optically active isomeric forms of lactic acid, D(-) and L(+). This compound is utilized across a range of industries including food, chemical, pharmaceutical and cosmetic. Lactic acid is amenable to several chemical conversions and used as a precursor for various chemicals and materials [4]. Lactic acid can be obtained by chemical synthesis leading to production of a racemic mixture, inevitably, while pure enantiomers, L (+) or D (-) lactic acid can be produced by fermentative process. L (+) lactic acid industrial production is extensively reported using several substrates, however, there are few studies related to commercial production of D(-) lactic acid [5].

Currently, lactic acid is becoming important as an intermediate feedstock use as a monomer for the synthesis of biodegradable polymers [6]. Lactic acid polymer (PLA) can be presented in three different forms namely, poly L-lactic acid, poly D-lactic acid, and poly DL-lactic acid [7]. PLA is being used in biomedical area in the form of implants, suture, bone fixation material and drug delivery microsphere due to its excellent biocompatibility and biodegradability [8,9].

For the production of lactic acid, the most important carbohydrates are the disaccharides lactose, maltose and sucrose [10]. In general, sucrose enters the cells also by a specific permease system, and it is split into glucose and fructose [11]. Since PLA manufacturers require large amount of lactic acid at a relatively low cost, an attempt to screen cheap raw materials is important to the feasibility of the microbial production of lactic acid [12]. However, when raw materials are used as a carbon source, they must be passed by a hydrolysis in order to make the sugars assimilable to the bacteria [13]. On the other hand, pure sugars usually facilitate the downstream processing, while cheap raw materials as alternative substrates result in the increase of downstream processing cost, due to their relatively high amount of impurities [14].

Rate of fermentation depends mainly on the parameters such as pH, temperature, agitation initial substrate concentration and concentration of nitrogenous nutrients [15]. Batch fermentation is widely used in lactic acid production, however, the long fermentation times required from this technic results in low productivity, as well low cell concentrations. Inhibitory effects of initial substrate concentration on production are also considered major bottlenecks of this fermentation manner. In order to solve these problems, fed-batch fermentation has been investigated to improve the production, productivity and yield of lactic acid fermentation. [3].

Lactic acid production is reported using several microorganisms, mainly lactic acid bacteria, including genetically modified strains, and some fung [16-19]. The species *S. nakayamae* belong to the genus *Sporolactobacillus*, the only genus of the family of Sporolactobacillaceae. It is a gram-positive bacterium endospore forming, microaerophilic and mesophilic, homofermentative that exclusively produces D(-) lactic acid [20]. The aims of this study were investigate the influence of concentration components of cultura media, as well the effects of temperature, pH, inoculum percentage, agitation conditions and pH controlling agents on the D(-) lactic acid production by *S. nakayamae.* Also fed-batch feeding strategies were studied in order to increase production and productivity levels of D(-) Lactic acid.

1. Materials and Methods

2.1 Bacteria Strain and culture medium

The producer strain was the previously indentified *as Sporolactobacillus nakayamae* by Macrogen (Korea). The GYP medium containing 20% (v/v) glycerol was used for maintenance of the strains at -80 ºC. The cells were propagated three times in GYP broth before use, incubated at 35±1 °C on stationary condition until the OD_{600} reached 2.5 (about 24 h).

2.2 Optimization of fermentation media

2.2.1 Response Surface Methodology (RSM)

RSM is a collection of experimental strategies, mathematical methods, and statistical inference which allowed efficient empirical exploration, with a reduced number of experiments [21]. In order to evaluate the influence of the crystallized sugar, yeast extract and GYP Salts solution (4 % $MgSO_4$. $7H_2O$, 0.16 % $MnSO_4$. $4H_2O$, 0.2 % $FeSO_4$. $7H_2O$ and 0.2 % NaCl) on lactic acid production by the *S. nakayamae*, a RSM was used, with 3 replicates at the center point, totaling 17 experiments. The medium used for fermentation was formulated based on the GYP medium used previously. The crystallized sugar it's a kind of commercial table sugar (sucrose) not very refined, found at an affordable price in Brazil. The yeast extract used is from Zilor (a major ethanol producer company in Brazil) this yeast extract it is a byproduct of ethanol fermentation, which is processed and available for a very low cost. The variables of the experiments were coded according to the equation A:

Equation A:

$$xi = (X_i - X_{cp})/\Delta X_i$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_{cp} is the real value of an independent variable at the center point, and ΔXi is the step change value. The levels used for encoding independent variables are shown in Table 1:

The fermentation runs were carried out in 125 mL Erlenmeyer flasks with 10% of inoculum, initial pH at 6.0 and 5% of $CaCO_3$ used as pH neutralizer agent. The flasks were incubated for 48 hours, at 35 °C in stationary condition. In the end of fermentation D(-) Lactic acid and residual sugar were quantified. In order to validate the optimization of the medium composition, five repetitions were carried out using the optimized condition (120 g/L of crystallized sugar, 35 g/L of yeast extract and 7.5 mL/L of GYP salt solution) to confirm the results of the response surface analysis, the results were presented as mean values. The experimental design was determined using the statistica 7 software program (StatSoft, Tulsa, USA) as well the results analysis on the experimental design.

2.3 Effect of Culture conditions on lactic acid production

Different process parameters such as pH controlling agent, temperature, pH, agitation and inoculum size, were optimized by varying the respective parameters to enhance D(-) lactic acid production. The fermentations were carried on bioreactor Infors HT Multifors 2 with a work volume of 300 mL of modified GYP medium (35 g/L yeast extract, 120 g/L crystallized sugar and 7.5 mL/L GYP salts), 10% (v/v) of inoculum (except on the test related with influence of inoculum size on lactic acid production) . The influence of pH controlling agent on D(-) lactic acid production was evaluated along of fermentation by automatic addition of controlling agents. The effect of pH on lactic acid production was evaluated from 5.0-7.0, while the influence of temperature was verified from 31 ºC to 39 ºC. To find the optimum agitation, the fermentation was carried under 0 - 150 rpm. The production medium was inoculated with some inoculum levels (5% - 25% v/v) . The fermentation was stopped after 48 hours. Samples were withdrawn periodically from the bioreactor to determine the concentrations of sucrose and D(-) lactic acid. The final volume of fermentation was considered to lactic acid normalization calculation (Normalization = production g/L x final volume mL/ initial volume mL).

2.4 Feed-batch fermentation strategies

Fed-batch fermentations were performed in a 750 mL bioreactor (Infors HT Multifors 2) with a working volume of 300 mL of optimized medium containing 35 g/L yeast extract, 7.5 mL/L GYP salts and various amounts of crystallized sugar as required. The fermentations were run at 35 ºC, 125 rpm, an inoculum volume of 20% (v/v) and NaOH was automatically added to control the pH at 6.0. The feed solution contained 730 g/L of crystallized sugar and 1% of yeast extract and in the fed-batch fermentation, the feed solution was pumped into the fermenter using a computer coupled peristaltic pump. The samples were withdrawn at desired intervals for further analysis to check the lactic acid production, sugar consumption, and cell growth. Batch fermentation was carried out at initial crystallized sugar concentration of 120 g/L, throughout the process, just NaOH was added to control pH.

Some feeding strategies were used for improving lactic acid production. In single pulse fed-batch fermentation, the initial concentration of crystallized sugar was 120 g/L. The feed solution was supplied once when the residual sugar concentration decreased to 60 g/L, feeding to bring the sugar concentration approximately to initial value, at 18 hours. Multi-pulse fed-batch fermentation was carried at an initial crystallized sugar concentration of 60 g/L. A pulse-feeding strategy was used by several additions of feeding solution when the residual sugar concentration was below 40 g/L.

In constant feed rate fed-batch fermentation, the feed solution was pumped into the fermenter at a feeding rate of 3 mL/h, when the residual sugar concentration decreased from initial 120 g/L to 60 g/L, started after 18 hours until 30 hours.

In exponential fed-batch fermentation, using Irirs 6 software, was applied a control strategy developed for Nor et al., 2011[22]. The rate of the feeding (F) was determined by equation B, which takes into account a mass balance, assuming a constant cell yield on substrate as well a constant maintenance coefficient throughout the fermentation.

Equation B:

$$F = \left(\frac{\mu}{Y_{X/S}(Si - S)}\right) V_0 X_0 \exp(\mu t)$$

Where *S* and *Si* are crystallized sugar concentration in medium at the beginning of the feed (g/l), and in feeding solution (g/l), respectively, *t* is culture time feeding (h), V_0 is culture initial volume (L), *X* and X_0 are cell concentration at start of feed (g/l), and initial cell concentration (g/l), respectively, $Y_{X/S}$ is cell yield on sucrose (g

cell/g crystallized sugar) and μ is cell specific growth rate (h^{-1}). Feeding was started after 18 hours and lasted 10 hours.

2.5 Analytical techniques

The fermented broth was used for the determination of D(-) lactic acid and residual sucrose. The samples were centrifuged at 7000·g, for 15 minutes, the supernatant was filtered through a 0.22 μm membrane and used for analysis. The concentrations were determined by high performance liquid chromatography (HPLC) equipped with a column Rezex ROA 300 mm x 7.8 mm (Phenomenex, USA), and differential refracting index detector (RID-A, Shimadzu). The mobile phase (0.005 M H_2SO_4), was fed at a flow rate of 0.6 mL/min and temperature was kept 65 °C. After media optimization process, the optical purity of lactic acid produced was determined by HPLC using a Chirex 3126 phenomenex (150 × 4.6 mm) column with 1 mM of CuSO4 as the mobile phase at 1 mL/min (26 °C). Cell growth was determined from the turbidity at 600 nm, the absorbance of the sample measured in a spectrophotometer was correlated to either the dry weight mass.

3. Results and discussion

3.1 Influence of the concentration components medium on lactic acid production by Response Surface Methodology (RSM)

In order to obtain higher lactic acid production, the culture conditions were optimized through a factorial design. The independent variables selected for the study of lactic acid production were: crystallized sugar, yeast extract, and GYP salts solution, distributed in two levels, two axial points and three central points. The results obtained after fermentation were expressed in g/l for the amount of lactic acid produced and residual sugar, in g/g for Yield and g/L.h for productivity (Table 2).

After 48 hour of fermentation, the highest production verified was 88.24 g/L of D(-) lactic acid (run10). In this run the residual sucrose was 25.49 g/L, also is verified a productivity of 1.84 g/Lh and a yield of 0.93 g/g.

A summary of the analysis of variance (ANOVA) for the selected quadratic model was shown in Table 3. The value of the determination coefficient R^2 (0.84) suggested that model can explain 84% of the total variation in response. The model is significant as evident from Fisher's test (Fcalc (4.39) > Ft (3.68)) and has a very low probability value Pmodel = 0.03. Among the model terms, the interactions X_1X_1 and X_2X_2 were

significant with a probability of 95%, demonstrating negative effect, probably due to catabolite repression by the substrates.

The results obtained were submitted to multiple regression analysis methods yielded the following regression equation (C):

Equation C:

$$Y = 85.34 + 3.93X_1 - 9.62X_1X_1 + 3.44X_2 - 13.78X_2X_2 - 0.02X_3 - 2.88X_3X_3 - 4{,}89X_1X_2 + 4{,}89X_1X_3 + 5.01X_2X_3$$

in which Y is the predicted response (D(-) lactic acid production) and X_1, X_2, X_3 are, respectively, the coded values of the test variables from crystallized sugar, yeast extract and salts solution.

The response for the regression equation was plotted in Figure 1. These graphs shows the interaction of the variables and optimum levels for D(-) lactic acid production:

Based on response surface graphs, was verified that the components medium concentration that induce highest D(-) lactic acid production were: 120 g/L crystallized sugar, 35 g/L yeast extract and 7.5 mL/L salt solution. The concentration of crystallized sugar was selected considering the residual sucrose, which should not exceed 10 g/L aiming further purification. The validation of the model for lactic acid production optimization was carried according to the selected concentrations, performed in five replicates and results are presented as mean values. Under these conditions, responses obtained were; 89.69 g/L D(-) lactic acid, 1.87 g/Lh productivity, 0.96 g/g Yield and left 26.67 g/L of residual sucrose. These results confirming the validity and usefulness of the model equation. The optimized culture media was analyzed by HPLC using a chiral column, to verify the optical purity of lactic acid produced. It was quantified 98.97 % of D(-) lactic acid and 1.03 % of L(+) lactic acid, showing the high optically purity characteristic of *S. nakayamae*.

3.2 Effects of neutralizant agents on D(-) lactic acid production

On lactic acid fermentation there is a drop in pH, becoming the microorganism unable to continue the fermentation, for this reason neutralization is essential. There are several bases that can be used to this purpose, and they are closely related to the downstream processing [23]. The effect of different neutralizing agents, including KOH 10 N, $Ca(OH)_2$ 6 N, NaOH 10 N, $CaCO_3$ 5% and 6%, NH_4OH 27% solutions on lactic acid production were investigated. The fermentations were carried using the optimized culture medium, at 35 ºC, pH

6.0 controlled by neutralizing agents automatically added to the process, except the $CaCO_3$ that was added into the fermentation vessel at the beginning of the process. The fermentations were conducted for 48 hours.

Based on the data obtained in table 4 the neutralizing agent selected was NaOH considering the practicality of process and the maximal volumetric lactic acid production (103.71 g/L), productivity (2.16 g/L.h) and Yield (0.94 g/g). No residual sugar was observed in this experiment, that is interesting to further lactic acid purification and polimarization, Moreover, NaOH used as pH controller in fermentation process does not generate precipitated waste, so it is considered environmentally friendly [24]. On the other hand, ammoniacal solution was not an appropriate neutralizing agent, obtained only a low lactic acid concentration (16.05 g/L). This result was in accordance with those reported by other authors studying lactic acid production by *Rhizopus oryzae* achieving 24.9 g/L of lactic acid when used ammoniacal solution as neutralizant agent, because high concentration of ammonia could to be toxic to cell growth [25].

Calcium carbonate ($CaCO_3$) is often used as such a pH controlling agent [26] and in the present study yield the second highest D(-) lactic acid production (91.17 g/L) when used in the proportion of 5%. The proportion of 6% inhibited the microorganism growth. The low solubility of $CaCO_3$ in the fermentation broth could cause problems in the subsequent purification process, considering the most used method recovery that consumes lime as well sulphuric acid and also produces a large quantity of calcium sulphate sludge as solid waste [27].

When $Ca(OH)_2$ 6 N was used, 88.95 g/L of D(-) lactic acid was achieved, although this neutralizant agent be affordable, was necessary stirred the solution constantly using a magnetic plate/stove during the fermentation to prevent clogs in the base feeding tubes, resulting in extra cost to the process. In contrast, a previous studie presents 127 g/L of D(-) lactic acid production using $Ca(OH)_2$ as neutralizant agent in laboratory scale, this author reported that a lower osmotic pressure derived from this neutralizing base might be partially responsible for the facilitation of D-lactic acid fermentation [28].

As shown in figure 2, among all neutralizing agents, when used NaOH or KOH the process ended without any residual sugar. Besides the production of 84.45 g/L lactic acid using KOH neutralization, this agent is unaffordable for large scale production. The following figure shows the time course of lactic acid and residual sucrose concentration under the conditions of using different neutralizing agents, for 48 hours.

3.3 Effects of culture conditions on lactic acid production

3.3.1 Influence of temperature and pH on D(-) lactic acid production

The capacity of microorganisms to produce lactic acid is influenced by some conditions such as temperature, pH, agitation, and inoculum percentage. For this reason, optimization of these parameters is important for developing the process. Temperature and pH are very important parameters that affect the fermentation process [29]. D(-) lactic acid produced by the *S. nakayamae* was efficient achieved at pH 6.0, with an optimum temperature at 35 ºC (figure 3 ab) reaching 99.43 g/L lactic acid and low level of residual sucrose (3.45 g/L). The productivity and Yield presented in these conditions were 2.07 g/Lh and 0.91 g/g respectively (not shown). In contrast, other authors reported that the highest D-lactate concentration was obtained by strains of *Sporolactobacillus* sp. at 42 °C [30,31].

The temperature influences the activity of metabolic cell, most lactic acid bacteria which are responsible for the conversion of sugar to lactic acid are classified as thermophilic or mesophilic bacteria and usually have an optimum growth between 20 and 40 ºC [32].

According to other authors, the optimum pH for lactic acid production by microorganisms varies between 5.0 and 7.0, being dependent of the microorganism species [33]. In this study, the lactic acid production significantly decreased with when the pH was lower than 6.0 (figure 3 b). According to the current results, it was previously reported that 52.37 g/L of D(-) lactic acid by *Lactobacillus* sp. LMI8 was obtained when the fermentation was carried on pH 6.0 [34].

3.3.2 Effect of agitation and inoculum size on D(-) lactic acid fermentation

The influences of agitation and inoculum size on the D(-) lactic acid production were investigated. The results are shown in Figure 4. The high D(-) lactic acid production (107.67 g/L) was observed when the fermentation was submited to 125 rpm agitation, in this condition it was not observed residual sucrose. Also was achieved high productivity (2.24 g/Lh) and Yield (0.98 g/g) (not shown). Under stationary condition the lactic acid production was not effective (65.03 g/L) maybe for the insufficient homogenization of the culture medium. On the other hand, it was reported no difference on lactic acid production by *Lactobacillus casei* under stationary or 100 rpm agitation condition [35].

The maximum lactic acid concentration (113.73 g/L) and low residual sucrose (3.37 g/L) was obtained whith 20% (v/v) of inoculum on media culture. In this condition 2.37 g/Lh of productivity and 0.98 g/g of Yield were achieved (not shown). The low lactic acid production (82.34 g/L) at 5% (v/v) inoculum level could be

attributed to the low density of starter culture. Conversely, other author studying the influence of inoculum size on L(+) lactic acid production by *Rhizopus oryzae* ASC081 note that the lactic acid production decrease when inoculum size was lower than 10%, due to an inadequate enzymatic efficiency [36].

3.4 Fed batch fementation strategies

The fed-batch cultures were developed to maximize the production and productivity of D(-) lactic acid by *S. nakayamae*. The time courses of cell growth, sucrose consumption, and D(-) lactic acid production during the batch and fed batch cultivations (pulse and multi-pulse) are shown in Figure 5.

A maximum D(-) lactic acid production (126.64 g/L) was obtained at the end of the single pulse fed-batch (Figure 5b), in the same cultivation was observed the highest cell density (11.67 g / L), however, it took 78 hours to achieve such values presenting a low productivity of 1.62 g/Lh. Bai et al., (2016)[37] used pulse fed-batch to improve D (-) lactic acid productivity by *Sporolactobacillus inulinus* YBS1-5, achieving 107 g/L of lactic acid and 1.19 g/L.h productivity at 90 h.

The fed batch fermentation applying 2 pulses (Figure 5c) and initiated at low level of sucrose, showed promising results, presenting the second highest level of lactic acid production (122.64 g/L) at 54 hours, at this time the residual sugar concentration was 13.47 g/L. However, this process had the highest productivity than others, at 13 hours (3.65 g/L.h). A low level of initial sugar combined with feeding it during fermentation avoid inhibitory effects of sugar on lactic acid production, improving the process, similar results was presented by Bai et al., (2003) [38] studying fed-batch fermentation to L (+) lactic production from *Lactobacillus lactis*.

The multi-pulse fed-batch culture, applying three pulses (figure 5d), was not satisfactory, probably the amount of sugar that was added causes high osmotic pressure of the cells, resulting in plasmolysis, and decreasing the rate of fermentation as well the sugar utilization, this result is in accordance with Kotzamanidis et al., (2002) [39]. Other authors reported higher production when used multi-pulse strategy than a single pulse, by *Sporolactobacillus* sp. CASD [31].

The final D-lactic acid concentrations in the constant feed rate and exponential fed-batch were 119.03 g/L and 121.02 g/L, respectively (Figure 6 a-b). These strategies were not satisfactory too, because it remains more then 20 g/L of sucrose concentration. Despite the productivity presented low values at the end of the process, along these cultivations the higher productivity was 3.15 (g/L.h) to exponential at 24 hrs and 3.06 (g/L.h) to constant feed rate at 30 hrs. Similar result was found by Bernardo et al. (2016) [40] using constant feed

rate feeding to L(+) lactic acid production by *Lactobacillus rhamnosus* B103. Their report a slightly increase of lactic acid production, however a high residual substrate concentration at the end of the process was presented. Other authors reported an improvement of 56.5% in L(+) lactic acid production using exponential feeding comparing with the traditional batch culture achieving 157.5 g/L of L(+) lactic acid and 1.88 g/L.h of productivity by *Lactobacillus casei*, after 84 h fermentation [41].

Except for three feeding pulse strategy, all other fed-batch estrategies were able to increase the production and productivity of D(-) lactic acid compared with batch fermentation (figure 5a), were the maximum production and productivity reached 106.95 g/L and 2.67 g/L.h, respectively.

4. Conclusion

D(-) Lactic acid was successfully produced by *S. nakayamae* under optimization conditions using fermentation. The experimental design was very useful for determining the optimal concentrations of constituents that have significant effects on D(-) lactic acid production. This study has shown that NaOH is an effective environmentally friendly pH controller agent, and the effects of pH, temperature, agitation and size of inoculum had a significant influence on the production process. The fed-batch culture was able to increase the production and productivity of D(-) lactic acid. These results demonstrated that *S. nakayamae* is a promising strain for D (-) lactic acid production.

Acknowledgments

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Figure captions

Figure 1 – Response surface for lactic acid production by *S. nakayamae*. The interactions between (a) Yeast extract and Crystallized sugar, (b) Salts and Crystallized sugar and (c) Yeast extract and Salts.

Figure 2 - Time course of lactic acid and residual sucrose concentration under the conditions of using different neutralizing agents (KOH, $Ca(OH)_2$, NaOH, CaCO and NH_4OH). Culture conditions: GYP modified medium, at 35 ºC and pH 6.0 with 10% (v/v) inoculum under 100 rpm.

Figure 3 - Effect of (a) temperature and (b) pH on D(-) lactic acid production and sucrose consumption by *S. nakayamae*. Culture conditions: GYP modified medium (120 g/L crystallyzed sugar, 35 g/L yeast extract and 7.5 salt solution) with NaOH 10N as pH neutralizant agent. a) GYP modified medium at pH 6.0, under 100 rpm, with 10% inoculum; b) GYP modified medium at 35 ºC, under 100 rpm, with 10% inoculum.

Figure 4 – Effect of (a) agitation and (b) inoculum size on D(-) lactic acid production and sucrose consumption by *S. nakayamae*. Culture conditions: GYP modified medium (120 g/L crystallyzed sugar, 35 g/L yeast extract and 7.5 salt solution) with NaOH 10 N as pH neutralizant agent. a) GYP modified medium at 35 ºC, pH 6.0, with 10% inoculum; b) GYP modified medium at 35 ºC and pH 6.0 under 125 rpm.

Figure 5- Time courses of sucrose consumption, D(-) lactic acid production and cell growth in batch fermentation (a), pulse fed-batch (b) and multi-pulse fed-batch fermentation (c and d) by *S. nakayamae*. Experimental conditions: medium containing 35 g/L yeast extract, 7.5 mL/L GYP salts and crystallized sugar (as required); pH 6.0, controlling agent NaOH 10N, temperature 35 ºC, 125 rpm, 20% of inoculum (v/v).

Figure 6- Time courses of sucrose consumption, D(-) lactic acid production and cell growth in constant feed rate (a) and exponential fed-batch fermentation (b) by *S. nakayamae*. Experimental conditions: medium containing 35 g/L yeast extract, 7.5 mL/L GYP salts and crystallized sugar (as required); pH 6.0, controlling agent NaOH 10N, temperature 35 ºC, 125 rpm, 20% of inoculum (v/v).

Tables

Table 1 - Variables and their levels for D (-) lactic acid production under a RSM

Variable		Levels				
		-1,68	-1	0	1	1,68
Cristallyzed sugar (g/L)	X_1	52.8	80	120	160	187.2
Yeast extract (g/L)	X_2	1.4	15	35	55	68.6
GYP Salt solution (mL/L)	X_3	0.9	3	6	9	11.04

Table 2 - RSM design with real values and experimental results for lactic acid production, productivity, yield and residual sugar concentration

Run	Experimental factors			Response variable			
	Crystallized sugar (g/L)	Yeast extract (g/L)	Salt solution (mL/L)	Lactic acid	Productivity	Y p/s	Residual sugar
	X_1	X_2	X_3	g/L	g/Lh	g/g	g/L
1	80.0	15.0	3.0	65.13	1.36	0.81	0
2	160.0	15.0	3.0	59.02	1.23	0.75	81.41
3	80.0	55.0	3.0	61.32	1.28	0.97	17.00
4	160.0	55.0	3.0	58.36	1.22	0.88	93.34
5	80.0	15.0	9.0	26.64	0.56	0.97	52.44
6	160.0	15.0	9.0	62.84	1.31	0.84	85.00
7	80.0	55.0	9.0	65.59	1.37	0.99	14.03
8	160.0	55.0	9.0	59.51	1.24	0.82	87.35
9	120.0	35.0	0.9	71.01	1.48	0.98	47.20
10	120.0	35.0	11.0	88.24	1.84	0.93	25.49
11	120.0	1.40	6.0	44.10	0.92	0.98	74.86
12	120.0	68.5	6.0	53.53	1.12	0.99	65.84
13	52.8	35.0	6.0	50.86	1.06	0.96	0.00
14	187.2	35.0	6.0	70.31	1.46	0.77	96.34
15	120.0	35.0	6.0	87.98	1.83	0.98	30.64
16	120.0	35.0	6.0	81.85	1.71	0.94	33.34
17	120.0	35.0	6.0	85.33	1.78	0.89	24.35

X_1= Crystallized sugar; X_2 = Yeast extract; X_3= salts.solution. Culture conditions: 35ºC, Initial pH 6.0 neutralized by 5% $CaCO_3$, stationary fermentation, 10% inoculum. Fermentation was carried for 48 hours.

Table 3. ANOVA with estimated regression coefficients for D(-) lactic acid production

Source	Coefficient	Sum of squares	Degree of freedom	Mean square	F ratio	P
X_1	3.93	211.632	1	211.632	2.351	0.169
X_1X_1	-9.62	1043.505	1	1043.505	11.594	0.011*
X_2	3.44	161.814	1	161.814	1.798	0.221
X_2X_2	-13.78	2141.408	1	2141.408	23.794	0.001*
X_3	-0.02	0.005	1	0.005	0.000	0.994
X_3X_3	-2.88	94.113	1	94.113	1.045	0.340
X_1X_2	-4.89	191.395	1	191.395	2.126	0.188
X_1X_3	4.89	191.982	1	191.982	2.133	0.187
X_2X_3	5.01	200.901	1	200.901	2.232	0.178
Model	-	3557.58	9	395.28	4.39	0.03
Error	-	629.97	7	89.99	-	-
Lack of fit	-	611.07	5	122.21	12.93	0.07
Pure error	-	18.90	2	9.45	-	-
Total	-	4187.55	16	-	-	-

X_1= Crystallized sugar; X_2 = Yeast extract; X_3= salts.solution *Statistically significant at 95% of probability level.

Table 4. Comparison of D-lactic acid fermentation by S. nakayamae using different neutralization agents.

Neutralizing Agent	Price	Used on fermentation	Cost on fermentation	D(-) Lactic acid*	P	Yp/s	RS*
	R$/kg	g/300 mL	R$	g/L	g/L.h	g/g	g/L
$CaCO_3$ 5%	44.00	15.00	0.66	91.17	1.90	0.90	11.76
$Ca(OH)_2$	40.00	11.11	0.44	88.95	1.85	0.81	3.52
NaOH	51.00	12.00	0.61	103.71	2.16	0.94	0.00
KOH	86.00	5.61	0.48	84.45	1.98	0.79	0.00
NH_4OH	17.20	9.17	0.17	16.05	0.33	0.13	88.85
$CaCO_3$ 6%	44.00	18.00	0.79	37.46	0.78	0.85	65.99

P= Productivity; Yp/s= Yield; RS = residual sucrose

*The normalized lactic acid and sucrose titer was calculated from the measured titer (not shown) from the fermentation broth with the dilution ratio from the neutralizing agent used. Productivity and Yield were calculated considering normalized data.

Fig. 1

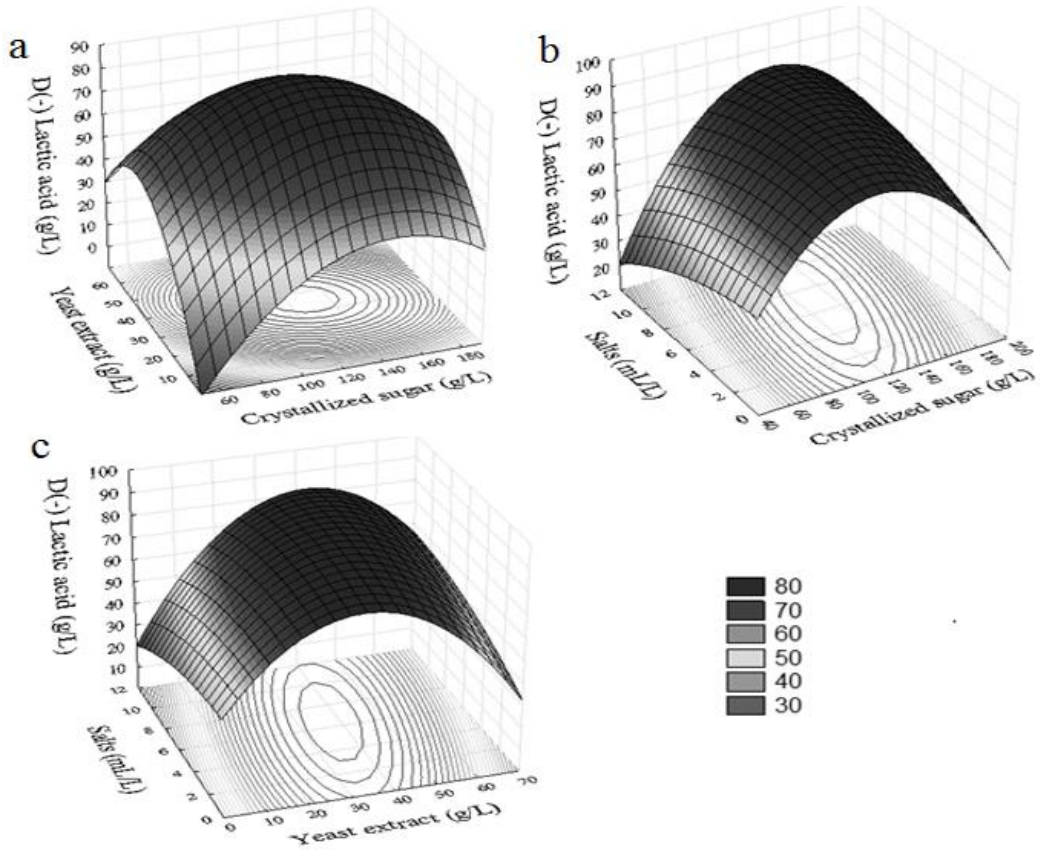

a
D(-) Lactic acid (g/L)
Yeast extract (g/L)
Crystallized sugar (g/L)
b
D(-) Lactic acid (g/L)
Salts (mL/L)
Crystallized sugar (g/L)
c
D(-) Lactic acid (g/L)
Salts (mL/L)
Yeast extract (g/L)
80
70
60
50
40
30

Fig. 2

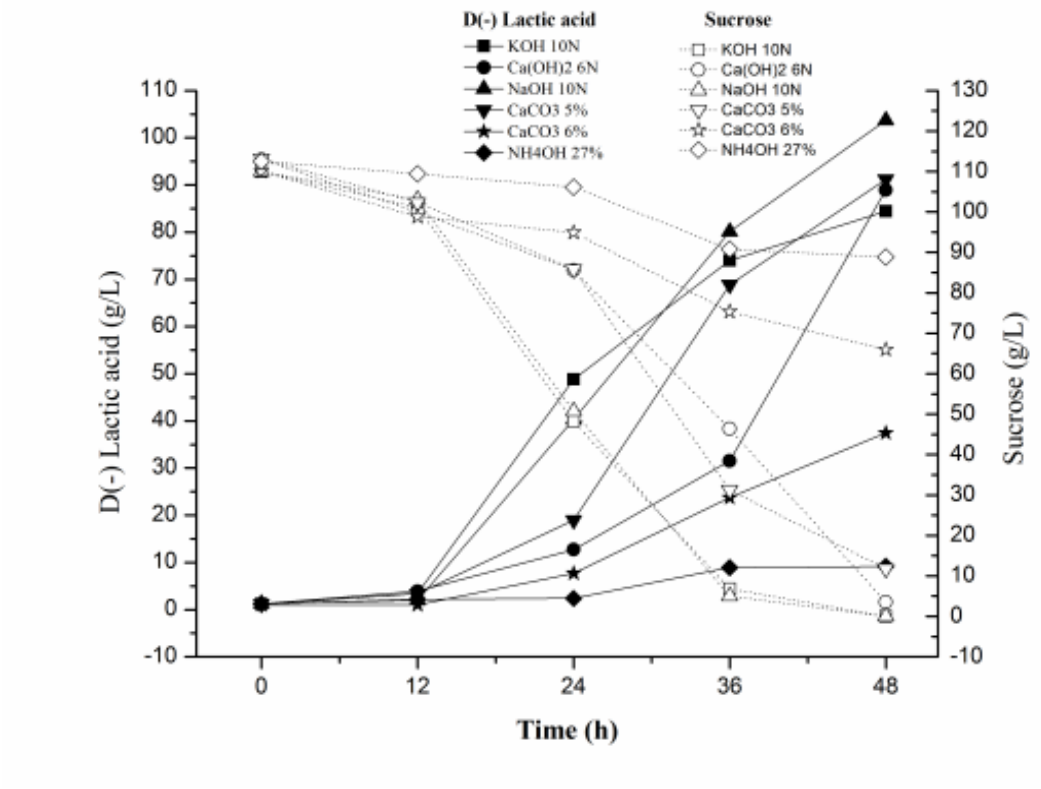

D(-) Lactic acid
KOH 10N
Ca(OH)2 6N
NaOH 10N
CaCO3 5%
CaCO3 6%
NH4OH 27%
Sucrose
KOH 10N
Ca(OH)2 6N
NaOH 10N
CaCO3 5%
CaCO3 6%
NH4OH 27%
D(-) Lactic acid (g/L)
Sucrose (g/L)
Time (h)
110
100
90
80
70
60
50
40
30
20
10
0
-10
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
0
12
24
36
48

Fig. 3

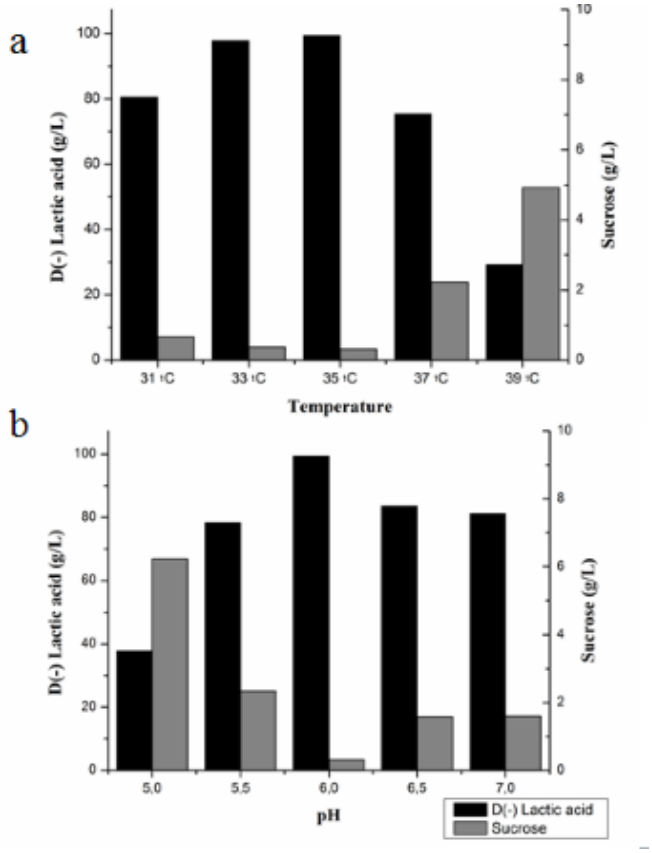

a
D(-) Lactic acid (g/L)
Sucrose (g/L)
31 ºC
33 ºC
35 ºC
37 ºC
39 ºC
Temperature
b
D(-) Lactic acid (g/L)
Sucrose (g/L)
5,0
5,5
6,0
6,5
7,0
pH
D(-) Lactic acid
Sucrose

Fig. 4

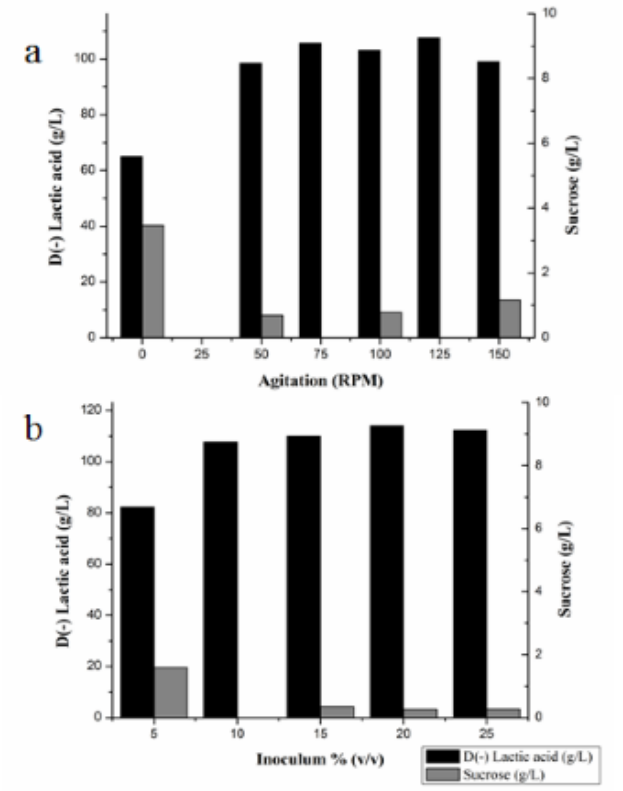

a
D(-) Lactic acid (g/L)
Sucrose (g/L)
Agitation (RPM)
b
D(-) Lactic acid (g/L)
Sucrose (g/L)
Inoculum % (v/v)
D(-) Lactic acid (g/L)
Sucrose (g/L)

Fig. 5

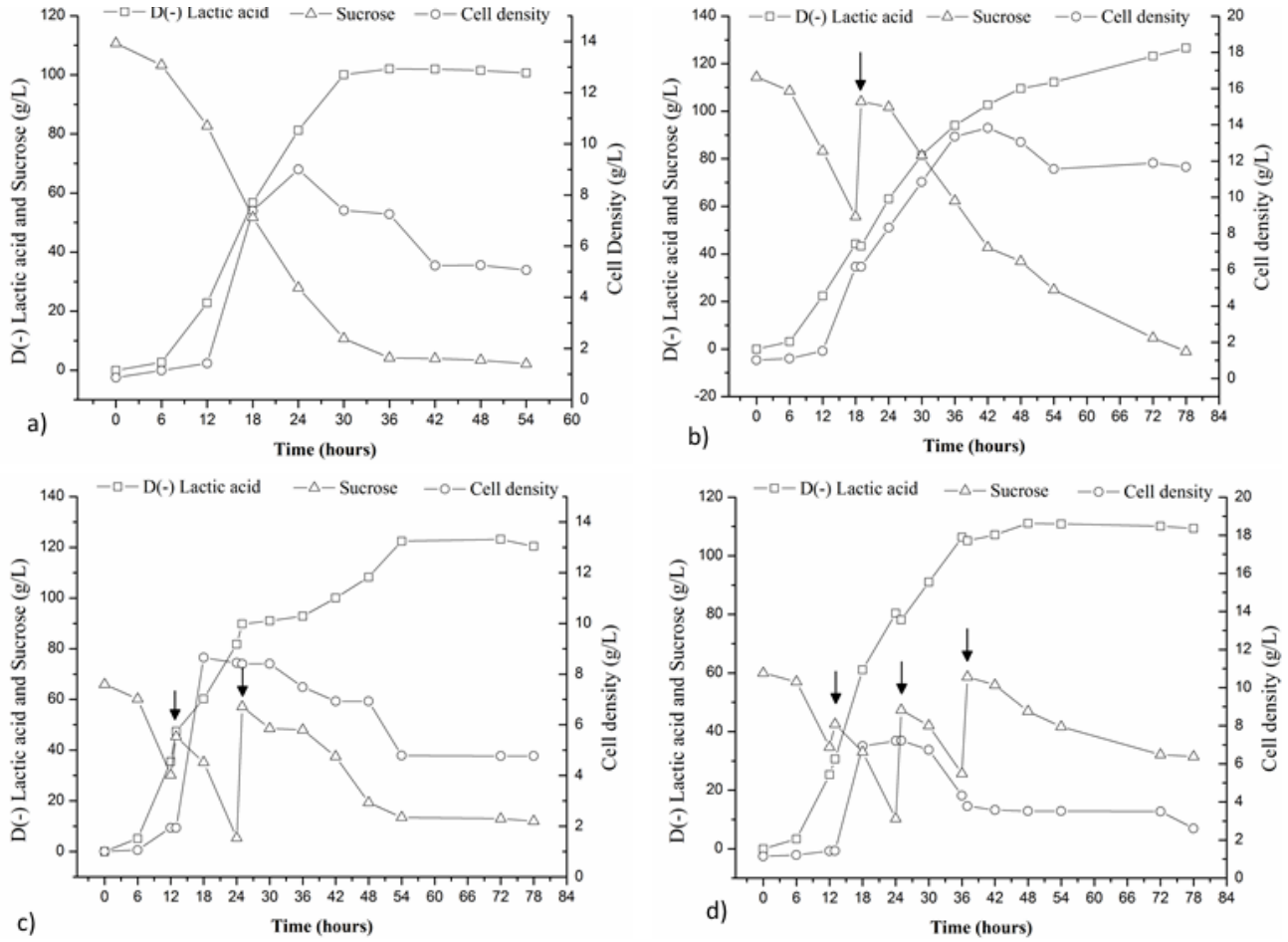
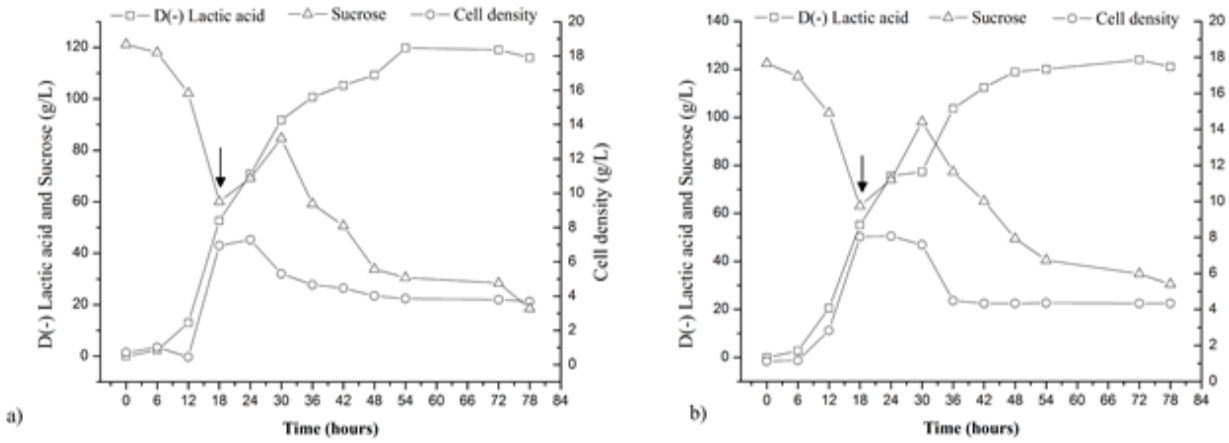

D(-) Lactic acid
Sucrose
Cell density
D(-) Lactic acid and Sucrose (g/L)
Cell Density (g/L)
Cell density (g/L)
Time (hours)
a)
b)
c)
d)

Fig. 6


D(-) Lactic acid
Sucrose
Cell density
D(-) Lactic acid and Sucrose (g/L)
Cell density (g/L)
Time (hours)
a)
b)

Capítulo III- Artigo submetido à revsita *Biomass & Bioenergy*

Efficient conversion of agro-industrial wastes into D(-) lactic acid by fed-batch fermentation using *Lactobacillus delbrueckii*

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Abstract

In this paper is described a green conversion of wastes such as molasses and corn step liquor into great levels of D(-) lactic acid, through a facilitated multi-pulse fed batch strategy using affordable pH neutralizer $Ca(OH)_2$. It's a very low cost process due no wastes hydrolysis were required. The fed-batch strategy could increase the lactic acid production and productivity avoiding inhibition by initial high substrate concentration and, allied to selected agent controller prevent cell stress that could be caused by high osmotic pressure of the culture media. It was also investigated effects of several carbon and nitrogen sources on lactic acid production, and, to determine the best concentration of components media, design experiments were applied. In order to optimize the culture conditions of the *Lactobacillus delbrueckii* strain, were examined the effects of pH control, temperature, neutralizing agent, agitation and inoculum size in batch cultures. In addition, fed-batch strategies were studied to improve the production and productivity. A high titer of D(-) lactic acid (162 g/liter) was achieved at 48 hours of fermentation, the productivity at this point is 3.37 g/L.h. It was found that the optimal conditions to run this process were 39 ºC, pH 5.5 controlled by addition of $Ca(OH)_2$, 150 rpm and 25% size of inoculum (v/v). The production of high optical purity D(-) lactic acid through *L. delbrueckii* fermentation from molasses and corn step liquor is a promising alternative economical process at industrial scale.

Keywords: D(-) lactic acid, agro-industrial wastes, *Lactobacillus delbrueckii*, fermentation, fed-batch

fermentation

1 Introduction

Lactic acid has gained considerable attention due of its role as a monomer for the production of biodegradable poly-lactic acid (PLA). Moreover is widely used in the food, cosmetic, pharmaceutical, leather, textile and chemical industries [1-4]. From long time ago until now, several methods have been studied on attempt to improve lactic acid fermentation with the goal on achieve highest yields [5].

There are two optical isomers of lactic acid, L(+) lactic acid and D(–). By microbial fermentation can be achieved optically pure L(+)- or D(–) lactic acid from appropriate microorganisms, which is capable to produce only one of the isomers selected. In the other hand, by chemical synthesis, a racemic DL-lactic acid is always produced [6-7]. Optical purity of lactic acid is an important physical property that have been attracting attention for industries, by blending L(+) or D(-) polymer together in order to polymerize high crystalline PLA, improving mechanical properties of this polymer [8-9].

There are numerous investigations on the development of L(+) lactic acid production of biotechnological process, therefore, this production in industrial scale has already been established [10-14]. However, D(-) lactic acid production by fermentation has fewer studies reported [15]. In industrial scale, lactic acid is usually produced by batch fermentation. However this method presents the disadvantage of product formation and productivity decrease due to inhibition by high substrate concentration [16]. In order to enhance the process productivity and avoid inhibitory effects of substrate on lactic acid production, different strategies of fed-batch fermentation have been explored [17-19].

Several lactic acid bacteria species belonging to the genera *Lactobacillus* such as *plantarum*, *delbrueckii*, *sakei*, casei and *lactis* have been widely reported on lactic acid production [19-23]. The production cost of lactic acid as well the demand to make it an environmentally feasible process, have a vital role on improvement of this technology [24]. Several studies presenting lactic acid production from row materials like rice starch, barley, wheat, corn, cottonseed meal, potato peel waste, fruit and vegetable wastes have been reported [25-29,17], however, these sources usually require pretreatment in order to release fermentable sugars, that is economically unfavorable [30]. The utilization of wastes from industrial process such as molasses that contains fermentable sugars, and corn step liquor, is a very economical alternative, these wastes can be readily used as nutritional sources on media fermentation decreasing total production costs [22, 30, 31].

Molasses sugar, a byproduct of the sugar industry, contains 40 to 60% sucrose, which can be converted to lactic acid by fermentation [6, 30]. Molasses is used mainly as carbon source and requires to be supplement with nitrogen and some minerals, in order to be used in fermentation media [32]. The corn step liquor is a waste generated in corn industry, containing amino acids, vitamins and minerals, which make it an excellent source of nitrogen that can used by microorganisms [33].

Nowadays, corn is the most produced cereal in the world, is expected a production of 989.30 million tones for the season 2015/2016, Brazil is among the three largest producers, along with China and USA [34]. Brazil is not only the largest sugarcane producer, it is also the world leader in the production of sugar. Along this process, a large amount of molasses is generated as the byproduct, produced in the proportion of 40 to 60 kilograms per ton of processed cane [35-36]. Looking for cost effective technologies, in this paper, it's described an efficient conversion of agro-industrial wastes (molasses and corn step liquor) into lactic acid by *L. delbrueckii* using fed-batch fermentation.

2 Material and Methods

2.1 Microorganisms and seed cultivation conditions

L. delbrueckii used in the present study was previously isolated from commercial yoghurt and it was stored in GYP (Glucose, yeast and peptone) medium with 20 % (by volume) glycerol at −80 °C. GYP medium is composed of 2 % glucose, 1 % yeast extract, 1 % peptone, 1 % sodium acetate, and 0.5 % (v/v) salt solution, which was comprised of 4 % $MgSO_4 \cdot 7H2O$, 0.16 % $MnSO_4 \cdot 4H_2O$, 0.2 % $FeSO4 \cdot 7H_2O$ and 0.2 % NaCl; pH 7.0. The seed medium was cultured in GYP medium for 15 h at 35 ºC on stationary conditions until OD_{600} reached 5.0 and then inoculated with 10% (v/v) into Erlenmeyer flasks or fermenter for D(-) lactic acid production (except on the test related with influence of inoculum size on lactic acid production).

2.2 Optimization of fermentation medium

2.2.1 Influence of Carbon and Nitrogen sources on lactic acid fermentation

The effect of different carbon sources on lactic acid production by *L. delbrueckii* was studied using molasses sugar, commercial sucrose and whey as substrate at concentration of 140 g/L of sugar content on GYP medium. After selected the carbon source, several nitrogen sources were evaluated, such as corn step liquor, Proflo, peanut flour, soybean flour, urea, ammonium sulfate, ammonium nitrate and yeast autolysate. The concentration of nitrogen sources were based on GYP medium. Fermentations were carried in 125 mL flasks containing 50 mL of medium at pH 7.0, 10% inoculum (v/v) on stationary condition at 35 ºC with incubation for 48 hours. To avoid pH decrease due to lactic acid production, 5% of $CaCO_3$ was added to each flask before sterilization. All experiments were carried out in duplicate to verify their reproducibility, and the results are expressed as mean values.

2.2.2 Response Surface Methodology (RSM)

After the sources that induced the highest lactic acid levels were selected, the carbon, nitrogen sources and salts concentrations were evaluated using the RSM, that is a collection of experimental strategies, used to find the optimum concentration of the medium components that had influence on D(−) lactic acid production. For this purpose, 17 experiments including 3 replicates at the center point were run. The independent variables selected for the study include the concentration of sugar molasses, corn step liquor and salts solution (the same used in GYP media).

The dependent variable (D(-) lactic acid production) was fitted by a second order equation and then generate the response surface graphics. Lactic acid fermentation was performed in the same conditions reported on the previous topic. The experimental design was determined using the statistica 7 software program (StatSoft, Tulsa, USA) as well the results analysis on the experimental design.

2.2.3 Study of D(-) lactic acid fermentation conditions

Fermentations were performed in a 750 mL bioreactor (Infors HT Multifors 2) with an initial working volume of 300 mL of optimized medium containing 150 g/L of molasses sugar (except for agitation and inoculum size experiments that beginning with 160 g/L), 65 mL/L of corn step liquor and 5 mL/L of salts. The system was equipped with pH, temperature sensors and pumps such as feed and base. The effects of pH controlling agent, temperature, pH, agitation and inoculum size on lactic acid production were investigated, as

shown below. The fermentation products were sampled periodically and analyzed to determine the concentrations of substrate, and lactic acid.

2.2.4 Neutralizing agents

Lactic acid produced along fermentation has to be continuously neutralized, the pH agent controller has influence on the lactic acid production as well as the final product. Acid neutralization on fermentation performance was investigated using different pH agent controllers such as $Ca(OH)_2$ 9N, NaOH 10N, NH_4OH 6N, $CaCO_3$ 5% and KOH 10N. The reactors were mechanically stirred at 100 rpm, and maintained at 35 ºC. The pH was automatic controlled at 6.0 by addition of each agents neutralizing. Cultivation medium (270 mL) was inoculated with 30 mL of standardized culture.

2.2.5 Parameters effect on D(-) lactic acid production

A series of experiments were carried to evaluate the effects of temperature, pH, agitation and inoculum size on lactic acid fermentation. Cultivation parameters used in these experiments are given in Table 1.

Table 3. Culture conditions on study of the parameters effect on lactic acid production

Parameter	T ºC	pH	Agitation rpm	Inoculum %	Change rate
Temperature range	**31-41**	6.0	100	10	2 ºC
pH range	39	**5.0 – 7.0**	100	10	0.5 units
Agitation range	39	5.5	**50-150**	10	25 rpm
Inoculum size range	39	5.5	150	**5-30**	5%

The fermentations were carried for 70 hours and $Ca(OH)_2$ 9N was used as pH agent controller.

2.2.6 Fed-batch fermentations

Fed-batch fermentation is used to prevent substrate inhibition by supplying nutrients either intermittently or continuously to the bioreactor along cultivation, keeping the cells and products there until the end of process [37,38].

In this study, different fed-batch fermentation of *L. delbrueckii* were implemented by pulse, multi-pulse and continuous feeding rate strategies. The fermentations were carried on a 750 mL bioreactor (Infors HT Multifors 2) with initial media containing 65 mL/L of corn step liquor, 5 mL/L of salts solutions (the same of GYP medium) and different content of sugar molasses, as described below. The processes run as the previously optimized conditions, at 39 ºC, 150 rpm, pH 5.5 controlled by automatic addition of $Ca(OH)_2$ 9N, the initial work volume was 300 mL considering 25% of inoculum (v/v). The feed solution was composed of 585 g/L of sucrose from molasses and 65 mL/L of corn step liquor that was pumped into the bioreactor by a computer coupled peristaltic pump, as required. In order to compare the processes, batch fermentation was performed on the same culture conditions containing 160 g/L of initial sucrose from molasses, being added during the process only the pH agent controller.

In pulse fed-batch process, initial molasses sugar concentration was set to 100 g/L, after 15 hours from beginning, when no residual sucrose was presented, a pulse was applied into bioreactor adding 60 g/L of sucrose

from feed solution. Still studying a single pulse strategy, other fermentation was carried starting the process with 150 g/L of molasses sugar, applying a pulse (55 g/L of sucrose) at a moment that the residual sugar was near 25 g/L. The multi pulse fed-batch process was started with 100 g/L of sugar molasses and the feed solution was supplied twice, when the residual sugar was below 10 g/L, at 15 hours and 24 hours, has been added 45 g/L and 30 g/L of sucrose from feed solution, respectively. In constant feed rate fed-batch fermentation, when all initial sucrose was consumed (at 15 hours), the feed solution was continuously pumped to the bioreactor at a feed rate of 7.58 mL/h, until 24 hours of fermentation. The fermentations were conducted through 70 hours, samples were collected periodically to verify the lactic acid and sugar concentrations, as well as cell growth.

3 Analysis of sugars, lactic acid and cell growth

At the end of fermentation, the samples were centrifuged at 7000 × g within 15 minutes, micro filtered through 0.22 μm membrane and then the supernatants were used to determine the concentration of residual sugar and D(-) lactic acid. The concentrations were determined by high performance liquid chromatography (HPLC) equipped with a column Rezex ROA 300 mm x 7.8 mm (Phenomenex, USA), and differential refracting index detector (RID-A, Shimadzu). The mobile phase (0.005 M H_2SO_4), was fed at a flow rate of 0.6 mL/min and temperature was kept 65 °C. The optical purity of lactic acid produced was determined by HPLC using a Chirex 3126 phenomenex (150 × 4.6 mm) column with 1 mM of CuSO4 as the mobile phase at 1 mL/min (26 °C). Cell concentration was determined by a calibration curve that correlating the optical density at 600 nm (measured on spectrophotometer) to the dry cell weight, which was determined by centrifugation at 7000 x g for 15 minutes, washing twice, first with HCl 0.3N and second with distilled water, than dried at 100 ºC for 24 hours.

4 Results and Discussion

4.1 Influence of carbon and nitrogen sources on D(-) lactic acid fermentation

L. delbrueckii was able to produce D(-) lactic acid using all the carbon sources studied, however the highest production (112.84 ± 3.07 g/L) was achieved on the presence of molasses sugar, presenting 12.12 ± 1.22 g/L of residual sucrose. Besides commercial sugar had induced similar lactic acid concentration (111.37 ± 1.15 g/L), it was verified largest amount of residual sucrose (24.98 ± 1.22) as showed on Table 2. Possibly molasses sugar provides a greater cell growth due to the presence of nitrogen in its composition. Also could be related to buffering action of molasses, once it contains calcium, as reported before by Dumbrepatil et al. (2008) [30].

Table 24. Influence of carbon and nitrogen sources on D(-) lactic acid production, productivity and Yield by *L. delbrueckii*

Carbon sources	Carbohydrates	D(-) Lactic acid (g/L)	Productivity (g/L.h)	Yield Yp/s (g/g)	Residual sugar (g/L)
Sugar molasses	46.9 (g/100 mL)[a]	112.84 ± 3.07	2.35 ± 0.06	0.88 ± 0.01	12.12 ± 1.22
Commercial sucrose	100 (g/100 g)[a]	111.37 ± 1.15	2.32 ± 0.02	0.96 ± 0.02	24.98 ± 1.22
Whey	76.59 (g/100 g)[b]	100.08 ± 2.06	2.08 ± 0.04	0.89 ± 0.02	28.32 ± 0.51
Nitrogen sources	**Percentage of N**				
Yeast autolyzate	7.2%	102.79 ± 1.92	2.14 ± 0.04	0.87 ± 0.00	22.08 ± 1.88
Corn step liquor	4.69%	101.06 ± 3.36	2.10 ± 0.07	0.86 ± 0.05	23.41 ± 1.85
Proflo	11.15%	75.74 ± 1.20	1.57 ± 0.03	0.75 ± 0.04	39.11 ± 4.04
Peanut flour	7.63%	66.03 ± 0.47	1.37 ± 0.01	0.68 ± 0.03	44.11 ± 1.84
Soybean flour	6.40%	62.65 ± 1.38	1.30 ± 0.03	0.71 ± 0.07	52.43 ± 3.47
Ammonium nitrate	34%	52.60 ± 3.40	1.09 ± 0.07	0.57 ± 0.13	48.92 ± 2.36
Urea	46.62%	49.16 ± 1.30	1.02 ± 0.03	0.66 ± 0.04	66.56 ± 1.07
Ammonium sulfate	21%	37.09 ± 0.10	0.77 ± 0.00	0.56 ± 0.03	74.46 ± 1.23

Culture conditions: GYP medium with 140 g/L of carbon source and 15.25% of nitrogen contained on each nitrogen sources, 5 % of $CaCO_3$, at 35 °C, stationary fermentation with an initial pH of 7.0 for 48 hours. Type of carbohydrate: a) Sucrose; b) Lactose

The molasses is a byproduct from the sugar industry, it's a syrupy material left after the removal of sugar from the mother syrup and are available in large amounts in Brazil, which is the biggest sugarcane producer as well as the first in the world on sugar production, responsible for more than half of the sugar commercialized in the world. The molasses provides the great advantage of present readily fermentable sucrose, not requiring pre-treatment and hydrolysis step [36, 39, 40]. Studies related with conversion molasses to lactic acid by *L. delbrueckii* have been reported [39, 40]. However some of them performed molasses hydrolysis with H_2SO_4 [30,41]. In the present paper *L. delbrueckii* was able to metabolize the sugar molasses without hydrolysis, offering more economy in the process.

Among the nitrogen sources studied, yeast autolyzate and corn step liquor induced a higher lactic acid production and productivity at the same rate, however the corn step liquor is a by-product from corn industry, for this reason it's a very cheap material. The use of agro-industrial wastes on fermentation configures environmental friendly process allowing the waste conversion into added-value products [42]. The lactic acid production was not favored by ammonium nitrate, ammonium sulfate and urea, once they are inorganic nitrogen sources, the culture media lacks of vitamins, which is important to microbial cell maintenance. Similar results are reported by (Assavasirijinda et al. 2016) [9] studying D(-) lactic acid production by an engineered *Bacillus* sp. strain, where inorganic nitrogen sources didn't induce satisfactory levels of production, however the highest lactate production was obtained using peanut meal as a nitrogen source. In this paper, higher lactic acid concentrations were verify when used wastes (Corn step liquor and ProFlo) that present some vitamins in their compositions such as Thiamine, Riboflavin, Niacin, Pantothenic acid, Choline, Pyridoxine, Biotin, Inositol, Carotene, Tacopherols, Ascorbic acid, Folic acid. Thus, molasses and corn steep liquor can be considered

alternatives substrates on lactic acid fermentation providing the nutrients requirements to *L. delbrueckii* used in this study.

4.2 Fermentation medium optimization by Response Surface Methodology

The mathematical relationship for the D(-) lactic acid production was developed by considering three independent variables that include some concentrations of sugar molasses, corn step liquor and salts solution; and one dependent variable: D(-) lactic acid concentration in final medium fermentation. Were also demonstrated the response values of productivity, Yield and residual sucrose for each run (Table 3).

Table 3. RSM design matrix with real/coded values and experimental results for lactic acid production, productivity, yield, and residual sugar concentration

Run	Dependent variables			D(-) lactic acid	Productivity	$Y_{p/s}$	Residual sucrose
	X_1	X_2	X_3	g/L	g/L.h	g/g	g/L
1	100.00 (-1)	35.00 (-1)	2.20 (-1)	82.30	1.71	0.86	21.66
2	200.00 (1)	35.00 (-1)	2.20 (-1)	82.61	1.72	0.73	107.22
3	100.00 (-1)	95.00 (1)	2.20 (-1)	79.53	1.66	0.95	32.40
4	200.00 (1)	95.00 (1)	2.20 (-1)	82.99	1.73	0.98	130.36
5	100.00 (-1)	35.00 (-1)	7.80 (1)	88.00	1.83	0.98	25.44
6	200.00 (1)	35.00 (-1)	7.80 (1)	85.35	1.78	0.91	122.48
7	100.00 (-1)	95.00 (1)	7.80 (1)	88.85	1.85	0.94	21.26
8	200.00 (1)	95.00 (1)	7.80 (1)	80.93	1.69	0.93	128.96
9	150.00 (0)	65.00 (0)	0.13 (-1.68)	102.03	2.13	0.97	60.27
10	150.00 (0)	65.00 (0)	9.87 (+1.68)	110.21	2.30	0.99	53.55
11	150.00 (0)	14.60 (-1.68)	5.00 (0)	82.39	1.72	0.97	80.36
12	150.00 (0)	115.40 (+1.68)	5.00 (0)	102.77	2.14	0.98	60.77
13	66.00 (-1.68)	65.00 (0)	5.00 (0)	68.88	1.44	0.97	10.61
14	234.00 (+1.68)	65.00 (0)	5.00 (0)	75.02	1.56	0.49	111.38
15	150.00 (0)	65.00 (0)	5.00 (0)	124.56	2.60	0.96	36.30
16	150.00 (0)	65.00 (0)	5.00 (0)	118.75	2.47	0.94	39.28
17	150.00 (0)	65.00 (0)	5.00 (0)	122.56	2.55	0.96	37.79

X_1= Sugar molasses (g/L of sucrose), X_2 = Corn step liquor (mL/L), X_3= Salts solution (mL/L). Culture conditions: Initial pH at 7.0, 5% of $CaCO_3$ used as pH neutralizer, 10% inoculum (v/v), and 35 ºC. Fermentation was carried under stationary condition for 48 hours. Coded values were present in parenthesis.

At the end of fermentation process, the highest titer of lactic acid (124.56 g/L) was achieved on run 15, as well the highest productivity (2.60 g/L.h) and residual sugar of 36.30 g/L, on this assay the central point values were used.

Analysis of variance for response surface quadratic model was represented in table 4.

Table 4. ANOVA for the regression model with estimated regression coefficients for D(-) lactic acid production

D(-) Lactic acid production					
Terms	Sum of squares	Degree of Freedom	Media of squares	F calc	*p*
Model	4505.71	9	500.63	13.08	0.001
Error	267.86	7	38.26	-	-
Lack of fit	250.44	5	50.08	5.74	0.154
Erro puro	17.42	2	8.71	-	-
Total	4773.57	16	-	-	-
$R^2 = 0.94$					
Terms		Coefficient			*p* value
Intercepto		122.24			0.0000*
X_1		0.25			0.8817
X_1X_1		-18.67			0.0000*
X_2		2.07			0.2554
X_2X_2		-11.37			0.0004*
X_3		2.15			0.2385
X_3X_3		-6.59			0.0090*
X_1X_2		-0.26			0.9069
X_1X_3		-1.79			0.4394
X_2X_3		-0.14			0.9481

X_1= Sugar molasses (g/L of sucrose), X_2 = Corn step liquor (mL/L), X_3= Salts solution (mL/L). *Statistically significant at 95% of probability level.

The regression equation presented R_2 value of 0.94, this value ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that the model could explain 94 % of the variability in the response. The model is significant as shown in Fisher's test Fcalc (13.08) > Ft (3.39) and has a very low probability value Pmodel = 0.001. Moreover, the Lack of fit value was not significant at 5% (p> 0.05), indicating satisfactory predictability of the model. The probability values (p) insure the significance of each coefficient, where *p* values <0.05 denote significant coefficient.

The interactions X_1X_1, X_2X_2 and X_3X_3 were significant with a probability of 95%, demonstrating negative effect. The high sucrose from molasses concentration can induce catabolic repression. High salt concentrations acts over membrane permeability of bacteria cell, as well as the osmotic pressure of the medium, may causes cell lysis or hindering the passage of essential nutrients through the microbial cell membrane. Corn step liquor is a waste that contained some salts in composition (Calcium (0.14%), Cooper (1,5 mg/100g), Manganese (2.0 mg.100g), Iron (10 mg/100g), Magnesium (0.6%), Potassium (2.8%), Sodium (0.1%), Phosphorus (1.8%)) among other impurities, which may combined with the salts solution provided could be too much to *L. delbrueckii* metabolized.

The results obtained were subjected to multiple linear regression analysis to yield the following regression equation (B):

Equation B:

$Y=122.24+0.25X_1-18.67X_1X_1+2.07X_2-11.37X_2X_2+2.15X_3-6.59X_3X_3-0.26X_1X_2-1.79X_1X_3-0.14X_2X_3$

Evaluations of the interactions between several factors which comprise a process are very important. RSM detects these interactions through three dimensional response surfaces, which demonstrate the effects of two factors on the response at a time and assist in arbitration of degree of parametric interactions on the desired responses [43]. The effects of the independent variables and their interactions on lactic acid production are illustrated in response surfaces graphics (Figure 1 a, b e c) constructed from equation generated according to the coefficient of linear regression model.

As shown in figure 1, maximal concentrations of lactic acid (120 g/L) was achieved when initial sucrose from molasses concentrations were approximately 150 g/L, corns step liquor concentration about 65 mL/L and salts solution concentration near 5 mL/L, these concentrations corresponding to central point values used on this experimental design. The lactic acid production is reduced when provided less than 60 g/L of corn step liquor, once in this process, the product formation is partially associated to cell growth, the nitrogen source plays an important role. This result is in accordance with Lima et al. (2009) [44] that applied RSM and achieved maximal D(-) lactic acid production (41.42 g/L) by *Lactobacillus* SMI8 using corn steep liquor and yeast autolysate, they notice that greater and lower nitrogen sources concentrations causes a reduction lactic acid production.

The lactic acid production increased with increases of molasses sugar concentrations up to 160 g/L, higher concentrations causes a decrease on lactic acid production probably due to substrate inhibition that is often reported on batch fermentations. Similar results were found by Dumbrepatil et al. (2008) [30] using a mutant *Lactobacillus delbrueckii* to produce lactic acid from molasses, reporting 166 g/L of lactic acid from 190 g/L of molasses sugar, when fermentation was carried under 240 g/L of sugar molasses was observed a substantial decrease in lactic acid production.

The salts studied could act as activators enzymatic, as reported by Lino et al. [21] that observed the lactic acid dehydrogenase activity was promoted when adding sodium acetate to the medium on lactic acid production by *Lactobacillus* species.

4.3 Effect of neutralizing agents on D(-) lactic acid production

The effect of different neutralizing agents on batch fermentations is shown in figure 2.

It was verified that the sucrose from molasses was completely consumed when the pH was controlled by $Ca(OH)_2$, yielding the highest lactic acid concentration of 111.02 g/L . On the presence $CaCO_3$ was achieved 98.82 g/L of lactic acid, however the residual sucrose is substantial (52.00 g/L). In the odder hand, when NaOH, NH_4OH and KOH were used, the sucrose wasn't completely converted into lactic acid by *L. delbrueckii* resulting in a low production. These results are in accordance with that reported by Liu et al, (2014) [45] when demonstrated that $Ca(OH)_2$ significantly facilitated D(-) lactic acid production compared to KOH or NH_4OH

using *Escherichia coli*. According to Nakano et al.(2012) [46] the pH of the medium was more efficiently neutralized by divalent cation (Ca^{2+}) compared to monovalent (Na^{+}, NH_3^{+}).

NaOH based fermentation is considering by some authors an environmentally friendly process that avoid generate precipitated waste [9,47], however this agent could increase the osmotic pressure of the medium, that causes osmotic stress to microbial cell. The osmotic stress influences cell growth and organic acid production during fermentation, causing inhibition effect mainly on the late stage of the process [48-49]. The neutralizing agents $Ca(OH)_2$ and $CaCO_3$ are generally applied on industrial lactic acid fermentation scale, because they makes the downstream process easier and cheaper when compared with other pH agents controller. Moreover they are also available at an affordable price than other [50].

4.4 Temperature and pH effect on lactic acid fermentation

Some important factors such as temperature, pH, agitation and inoculum size have to be considering for the efficient production of lactic acid. Temperature and pH are closely related with bacteria ability to grow, consequently affecting its production of lactic acid [51]. The pH affects the activity of enzymes and the transport of nutrients into the microbial cell, also had influence on RNA and protein synthesis [52]. In order to verify the best temperature of the process, the fermentations were carried out at 31 °C until 41°C. The optimum temperature to lactic acid production was observed at 39 ºC yielding 115.13 g/L, highest temperatures like 41 ºC as well lower temperatures such as 31 ºC and 33 ºC didn't assist satisfactory performance of *L. delbrueckii* on lactic acid production (figure 3 a).

According to results showed on figure 3b , the highest lactic acid production was observed when the pH was maintained to 5.5, reaching a concentration of 117.88 g/L. Higher pH such 6.5 and 7.0 presents a negative effect on D(-) lactic acid production (figure 3 b).

Lactobacillus genus comprises several species, which the optimum temperature for growth is between 40 °C and 35 ºC at pH between 5.5 and 6.0 [53]. Several studies using *Lactobacillus* sp. has been demonstrated that the best pH and temperature on lactic acid fermentation within the mentioned range. Wee et al. (2005) [54] reported that optimum temperature and pH for a batch culture of *Lactobacillus* sp. RKY2 was found to be 6.0 and 36 ºC respectively, achieving 153.9 g/L of lactic acid from glucose and yeast extract based media. Tang et al. (2016) [10] related the highest lactic acid yield (0.46 g/g) was obtained at 37 °C and pH 6 by *Lactobacillus* sp. Lima et al. (2010) [55] studying lactic acid produced from whey lactose and corn step liquor by *Lactobacillus* sp. LMI8 found the best temperature and pH at 39.6 °C and pH=5.9, respectively.

4.5 Effect of agitation and inoculum size on D(-) lactic acid production

The lactic acid production ability was investigated in some batch fermentation experiments varying the agitation speed and size of inoculum as shown in Figure 4 a and b.

To find the effect of inoculum size on lactic acid production, the optimized medium was inoculated with different percentage of culture seed (figure 4 b). The lower inoculum size (5%) and the higher (30%) didn't provide a satisfactory lactic acid production. It had been reported that small size of inoculum results on low conversion of lactic acid, due to an inadequate enzymatic efficiency [56, 57]. Similar result was reported by

Panesar et al, (2010) [52] verifying enhance of lactic acid formation and lactose utilization by *Lactobacillus casei* with an increase of inoculum size.

The best results on present study were verified when used 25% of inoculum size and 150 rpm, achieving 122.55 g/L and 127.35 g/L of D(-) lactic acid, respectively. Once the medium was composed by wastes, presenting several particles that tend to precipitate, a more vigorous agitation promote a greater homogenization, thereby increasing the contact of the cells with the components medium. Lower agitations could present an insufficient homogenization and the substrate could not be completely utilized, as verified when 50 rpm and 75 rpm were used. Indeed, no residual sugar was observed at the end of the process when were applied the highest levels of agitations. Moreover, agitation is important for mass transfer and heat transfer [58].

4.6 Study of fed-batch fermentations strategies

Through multi pulse fed batch strategy were achieved the highest values of D(-) lactic acid production (161.93 g/L) at 48 hours of fermentation, however at 30 hours was verified a production of 136.15 g/L of lactic acid yielding the best productivity of 4.54 g/L.h. This fermentation was performed under low initial sugar concentration and applying two pulses when the sugar content was almost or completely exhausted. This method allied pH controlled by $Ca(OH)_2$ avoid excessive osmotic pressure that medium components could causes into bacterial cell. By providing small quantities of sucrose from molasses on *L. delbrueckii* log phase, the inhibition of substrate was prevented. Fed batch strategy promotes an increase of 33.73% of lactic acid production comparing with batch culture. Many studies have been reported an improvement of the fermentation performance under fed batch fermentations. Assavasirijinda et al. (2016) [9] using a mutant *Bacillus* strain, growing in glucose and peanut meal based medium, performed a multi pulse fed-batch fermentation and reported a productivity of approximately 3.02 g/L.h at 16 h reaching a final D(-) lactic acid concentration of 142.05 g/l. Using the twice fed-batch feeding strategy, Li et al. (2013) [61] reported a 144.4 g/L of D(-) lactic production at 35 hours of fermentation as well as 4.13 g/L.h of productivity by *Sporolactobacillus laevolacticus* DSM442.

In table 5 was presented the best result for each fed-batch strategy, which was achieved at different times of fermentation.

Table 55. Fed-batch fermentation strategies on D(-) lactic acid production

	Batch culture	Pulse fed-batch I	Pulse fed-batch II	Multipulse fed-batch	Constant feed rate fed-batch
Fermentation time (h)	33	48	54	48	30
D(-) lactic acid (g/L)	121.08	138.84	139.42	161.93	118.79
Productivity (g/L.h)	3.36	2.89	2.58	3.37	3.96
Yield $Y_{p/s}$ (g/g)	0.61	0.78	0.55	0.81	0.61
Residual Sucrose (g/L)	0	0	7.97	0	31.63
Cell growth (g/L)	22.55	26.57	25.80	29.42	27.07
Final broth volume (mL)	400	520	480	580	550

Pulse fed-batch I started with 95 g/L of initial molasses sugar; Pulse fed-batch II started with 150 g/L of initial molasses sugar;

It's evident that supply a high substrate concentration at the beginning of the process is not advantageous as reported previously [59,60]. It's verified that the single pulse strategy, that was initiated with higher substrate content (pulse fed-batch II) took more time to reached the same concentration of lactic acid produced compared with pulse fed-batch where was supplied low initial substrate concentration (Fed-batch I). Similar result was presented by Ye et al. (2013) [59], that observed improve on productivity and production (3.2 g/L h and 140.4 g/L, respectively) in single pulse fed-batch compared with batch culture (2.6 g/L h and 118.2 g/l, respectively) by *Bacillus coagulans* C106.

In figure 5 were presented a kinetics of fed-batch fermentations, demonstrating the D(-) lactic acid production, sucrose from molasses consumption and cell growth rate.

The Constant feed rate fed-batch was not satisfactory, presenting the lowest D(-) lactic acid concentration among others. At the moment that was stopped to supply the feed, was observed microbial cells entering in stationary phase and declining moments after. This result is in accordance to Ding and Tan, (2006) [19] that didn't achieve good results applying this strategy on *Lactobacillus casei* fermentation**.**

The final culture media (from multi-pulse fed-batch fermentation) was analyzed to verify the optical purity of lactic acid produced, and it was observed a high optical purity of D(-) lactic acid (97.66% of D(-) and 2.34% of L(+) lactic acid).

5 Conclusion

The results demonstrated an efficient and low cost process to high optical purity D(-) lactic acid production by *L. delbrueckii,* which was able to grow and convert sugar from wastes such as molasses and corn step liquor into D(-) lactic acid. This is a very economic process once no pretreatment of the wastes were required. Great levels of lactic acid were achieved as well high productivity, moreover, the pH neutralized used presents the advantage of be available on very affordable costs and contribute to maintaining a low osmotic pressure on the culture media, that is very important to avoid stress on microbial cells. The fed-batch strategy by multi pulse, carried with the optimal conditions studied, is an easily and effective method that could improve the D(-) lactic acid production.

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Figure captions

Figure 1 - The interaction of media elements on lactic acid production using RSM. a) Corn step liquor and Salts solution; b) Corn step liquor and sugar molasses and c) Salts solution and sugar molasses.

Figure 2- Effect of neutralizing agents on D(-) Lactic acid production and residual sugar content by fermentation process with molasses sugar (150 g/L), corn step liquor (65 mL/L) and salts solution (5 mL/L). Culture conditions: 35 ºC, 100 rpm, 10% inoculum (v/v) and pH 6.0.

Figure 3- The effects of temperature (a) and pH (b) on lactic acid fermentation.

Figure 4- The effects of agitation (a) and inoculum size (b) on lactic acid fermentation.

Figure 5- Fed-batch fermentations of D(-) lactic acid by the *L. delbrueckii*. a and c) pulse; b) multi-pulse; d) continuous feed rate. Culture conditions: 39 ºC, 150 rpm, 25% inoculum (v/v) pH 5.5 controlled by $Ca(OH)_2$.

Figures

Fig1.

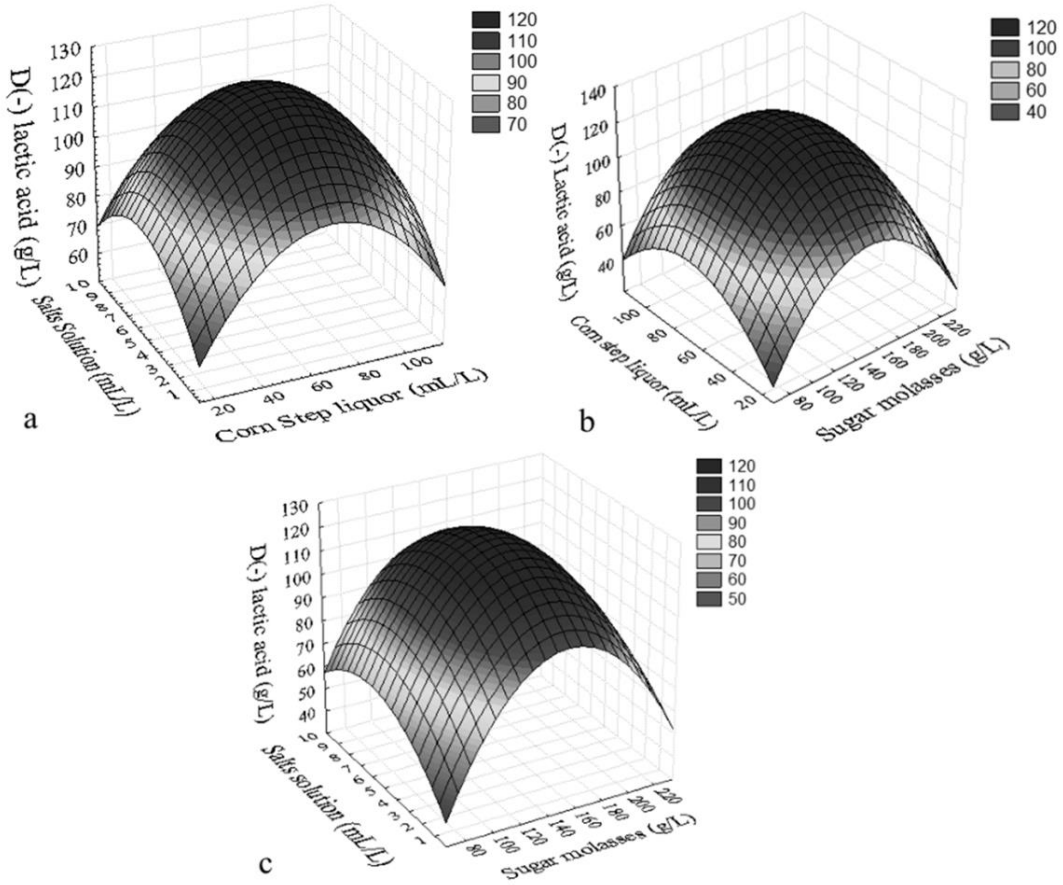


Fig 2.

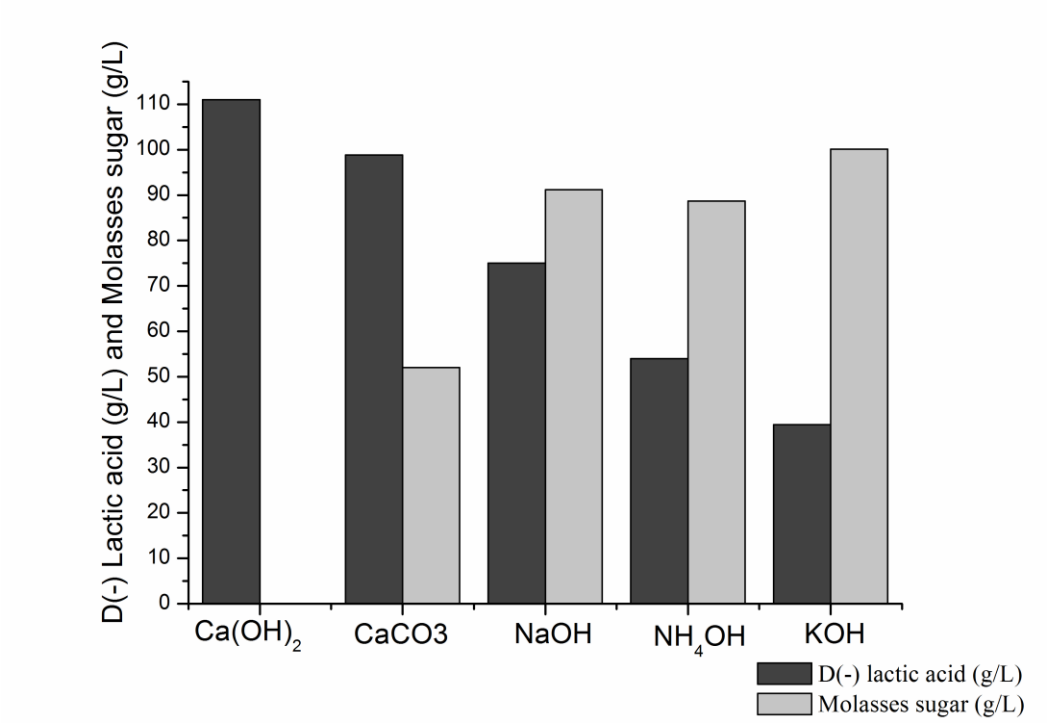

D(-) Lactic acid (g/L) and Molasses sugar (g/L)
0
10
20
30
40
50
60
70
80
90
100
110
Ca(OH)2
CaCO3
NaOH
NH4OH
KOH
D(-) lactic acid (g/L)
Molasses sugar (g/L)

Fig. 3

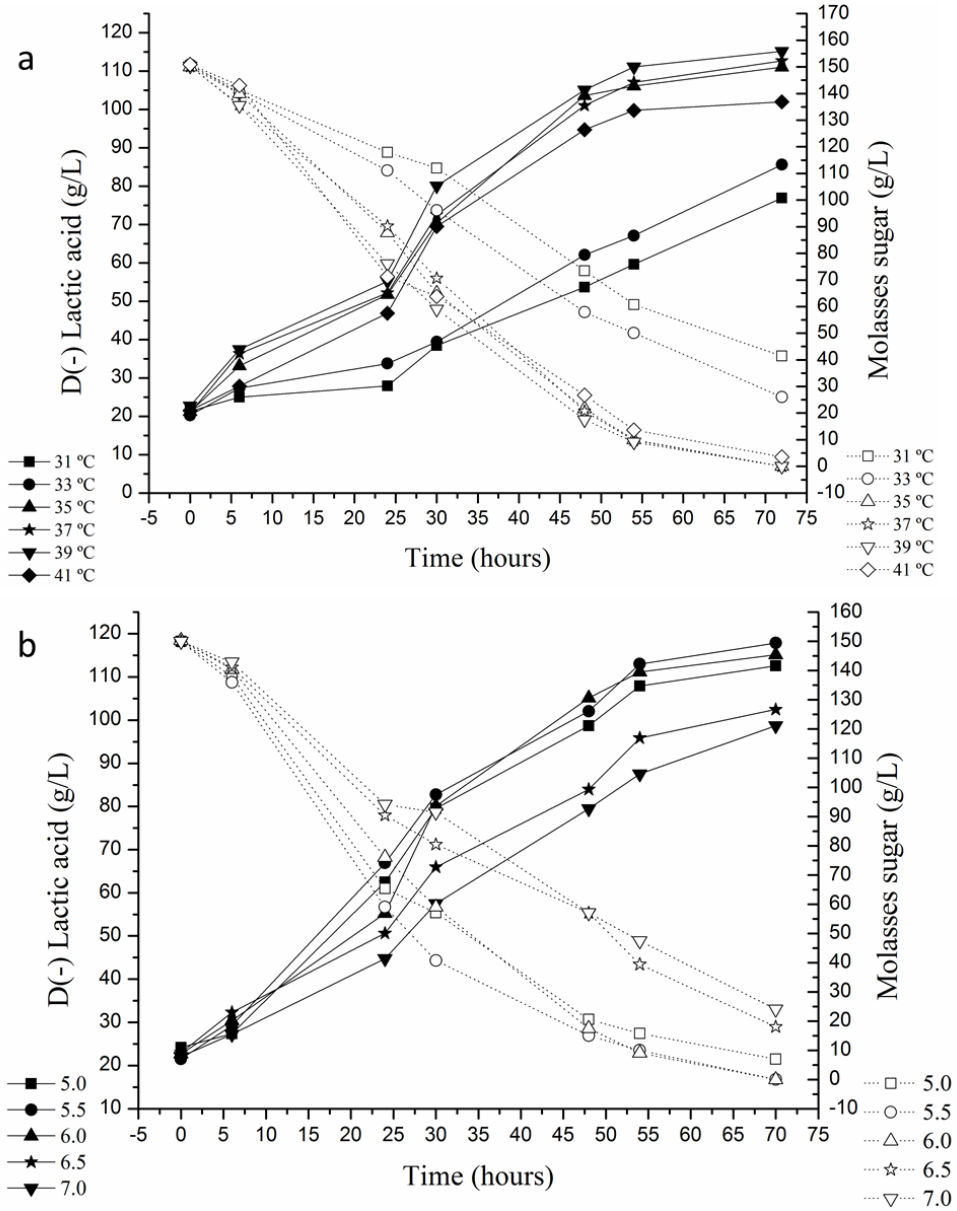

a
D(-) Lactic acid (g/L)
Molasses sugar (g/L)
Time (hours)
31 ºC
33 ºC
35 ºC
37 ºC
39 ºC
41 ºC
b
D(-) Lactic acid (g/L)
Molasses sugar (g/L)
Time (hours)
5.0
5.5
6.0
6.5
7.0

Fig. 4

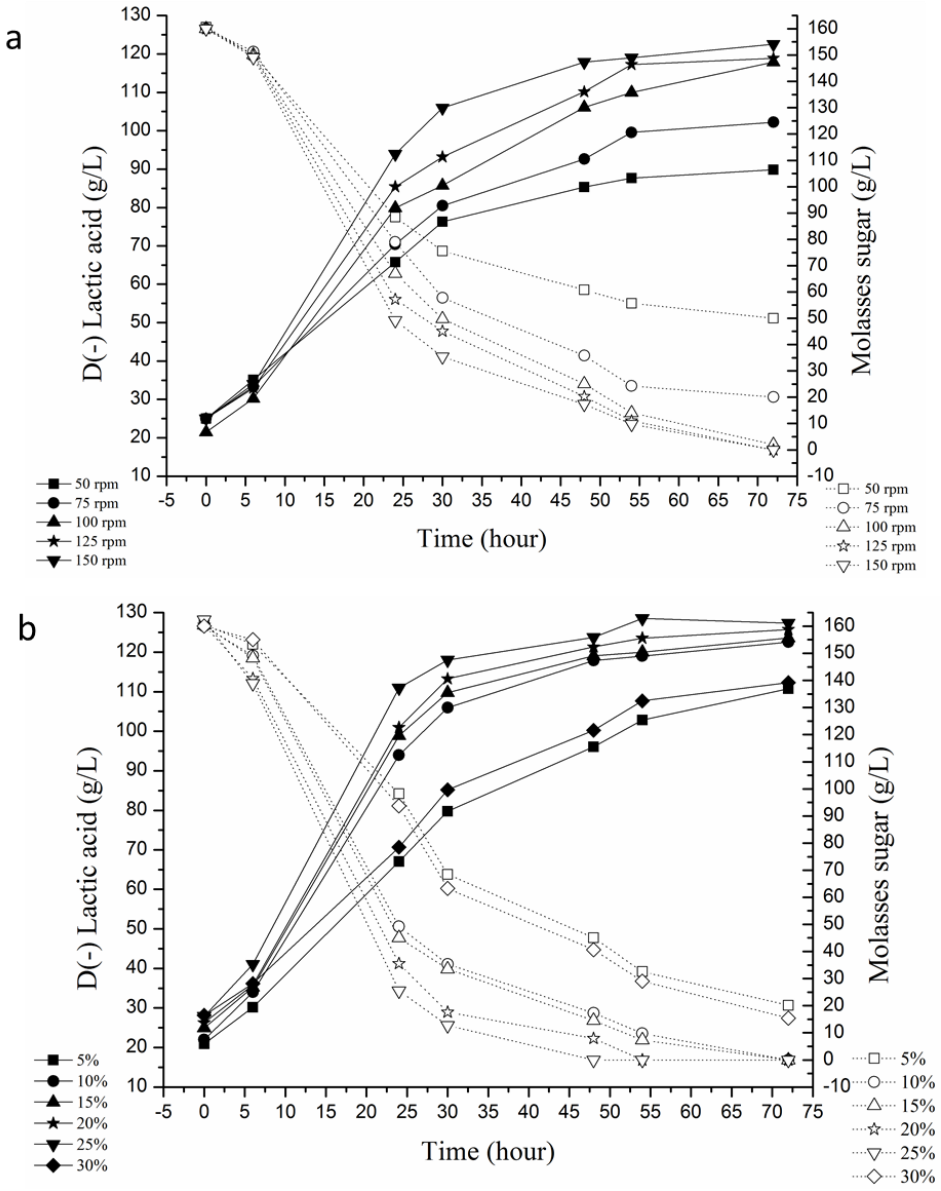
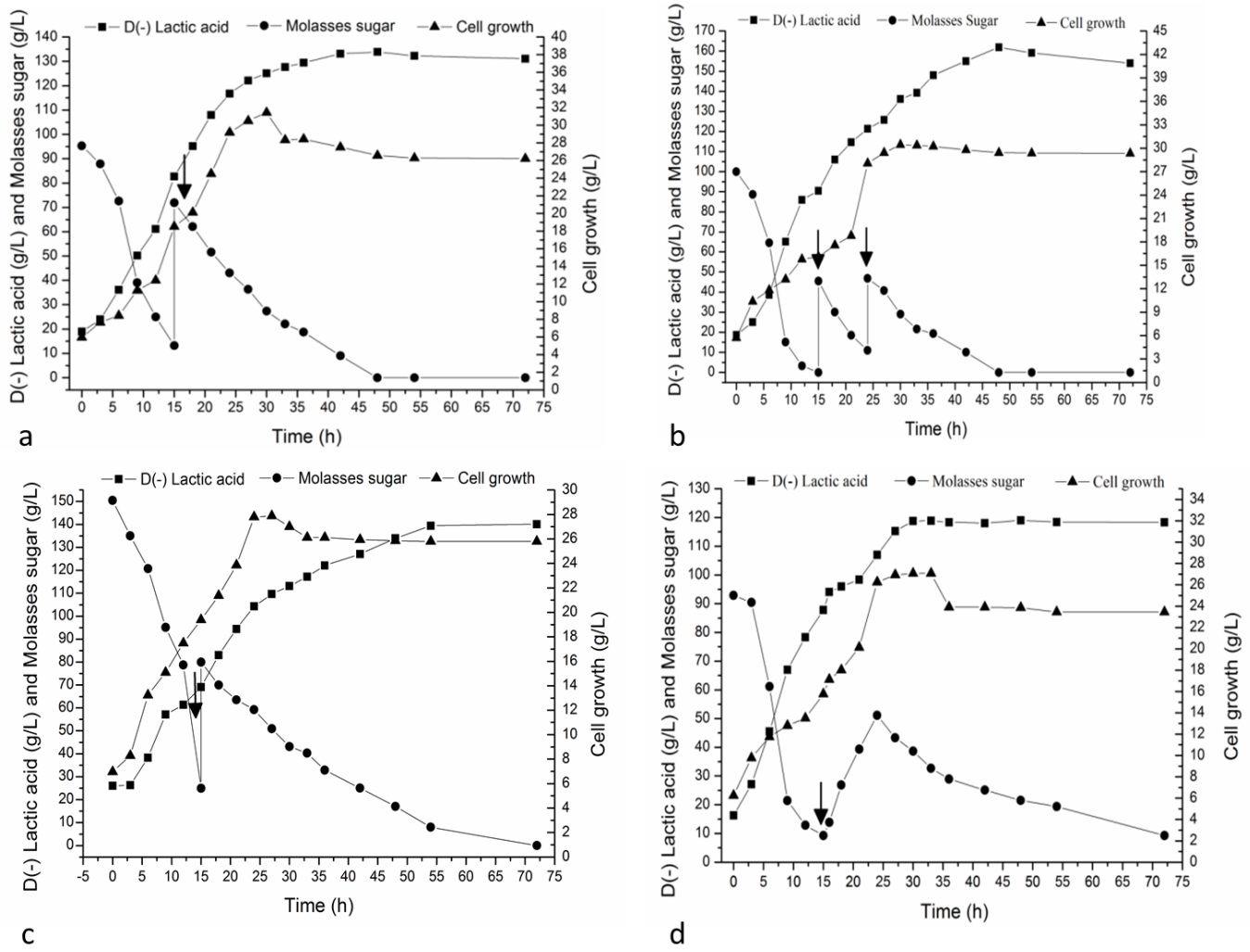

a
D(-) Lactic acid (g/L)
Molasses sugar (g/L)
Time (hour)
50 rpm
75 rpm
100 rpm
125 rpm
150 rpm
b
D(-) Lactic acid (g/L)
Molasses sugar (g/L)
Time (hour)
5%
10%
15%
20%
25%
30%

Fig. 5


D(-) Lactic acid
Molasses sugar
Cell growth
D(-) Lactic acid (g/L) and Molasses sugar (g/L)
Cell growth (g/L)
Time (h)
a
b
c
d

4 Considerações finais

Os micro-organismos *S. nakayamae* e *L. delbrueckii* foram capazes de produzir elevados níveis de D(-) ácido lático por fermentação. Quanto aos planejamentos experimentais, o Plackett-Burman e o fatorial fracionado foram úteis na seleção dos componentes significativos do meio de cultivo, porém observa-se que, esta técnica deve ser aliada ao bom senso do pesquisador, levando em consideração particularidades do processo e do metabolismo microbiano, a fim de interpretar corretamente os resultados. A metodologia de superfície de resposta apresentou-se muito eficiente na seleção das concentrações adequadas dos componentes do meio de fermentação. Foram apresentados dois perfis de produção distintos, um para cada micro-organismo. A metodologia de produção de ácido lático pelo *S. nakayamae* é baseada em um meio de cultivo mais puro, considerando o açúcar cristal e extrato de levedura utilizados como fonte de carbono e nitrogênio, respectivamente, apesar dessas fontes serem disponibilizadas por um custo acessível, ainda são mais dispendiosas que os resíduos agro-industriais. NaOH foi o controlador de pH utilizado neste processo, não é um dos controladores economicamente mais acessíveis porém oferece muita facilidade no processo por ser bastante solúvel, pode ser utilizado em altas concentrações, sem apresentar obstruções nas mangueiras que o conduzem até o interior do fermentador, assim como não aumentam demasiado o volume de trabalho do processo. Além disso, se for considerado um futuro processo de separação por eletrodiálise, o NaOH poderia ser reutilizado na fermentação. As condições de temperatura, pH e agitação estão de acordo com relatos da literatura para esta espécie e a estratégia de alimentação utilizada por um pulso oferece facilidade do processo.

A técnica de purificação utilizada foi eficiente na separação dos resíduos do processo e recuperação do ácido lático, além de ser conduzida de forma simplificada utilizando materiais não onerosos.

Portanto, trata-se de um processo que apresenta um custo moderado, porém compensado pela facilidade de condução como também no processo de *downstream*, por não apresentar muitas impurezas no meio fermentado final, facilita a purificação, assim como minimiza a geração de resíduos.

Ainda para este micro-organismo, o meio de cultivo utilizando farinha de amendoim como fonte de nitrogênio é uma alternativa econômica, porém para este modelo de reator

foram apresentadas dificuldades na condução do processo uma vez que o meio de cultivo se torna bastante consistente, obstruindo mangueiras na coleta de amostras.

Um segundo perfil de produção de ácido lático utilizando o *L. delbrueckii* como agente conversor apresenta uma produção mais econômica, baseada em resíduos agro-industriais, utilizando um neutralizador de pH $Ca(OH)_2$ muito acessível economicamente. Este micro-organismo apresenta a uma maior velocidade de crescimento e produção e foi capaz de produzir níveis superiores de ácido lático em relação ao *S. nakayamae*. Contudo, a purificação deste meio fermentado pode ser mais complexa, por conter impurezas oriundas dos resíduos agro-industriais utilizados, e se considerar o método de purificação usualmente utilizado nas indústrias para separação do Cálcio, a geração de resíduos deve ser considerada. Conclui-se que, os dois perfis são interessantes e oferecem diferentes possibilidades, que podem ser aproveitadas de acordo com determinados interesses e estruturas específicas operacionais para uma produção em maior escala.

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Anexo

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ORIGINAL ARTICLE

High D(−) lactic acid levels production by *Sporolactobacillus nakayamae* and an efficient purification

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Abstract This study is the first to report the efficient production of D(−) lactic acid by a strain of *Sporolactobacillus nakayamae* utilizing inexpensive peanut flour as a nitrogen source and commercial sucrose as a carbon source. A D(−) lactic acid concentration of 112.93 g/L was obtained by fermentation using 110.00 g/L of commercial sucrose, 150.40 g/L of peanut flour, and 0.16 ml/L of Tween 80. The optimal medium composition was determined using experimental design such as a Plackett-Burman, fractional factorial designs, and response surface methodology. The purification and recovery of D(−) lactic acid from the fermentation medium were performed using two purification techniques (filtration with activated carbon, followed by celite and cation resin exchange). This approach showed a high efficiency in protein and sugar removal as well in D(−) lactic acid recovery from the fermentation medium. Thus, the results indicate that *S. nakayamae* is a promising new D(−) lactic acid producer.

Keywords D(−) Lactic acid · Isomers · *Sporolactobacillus nakayamae* · Purification · Submerged fermentation

Introduction

The production of lactic acid by microorganisms has gained a prime position in industry because it is cost effective and eco-friendly. Lactic acid is a versatile chemical with a wide variety of applications across a range of industries (John et al. 2009; Djukić-Vuković et al. 2012). Lactic acid can be produced by fermentation from different raw materials and through various technological means; additionally, it has versatile properties as a precursor for various chemicals and materials (Kim et al. 2006; Zhang et al. 2007; Hetényi et al. 2008; Dumbrepatil et al. 2008).

Approximately 40 % of produced lactic acid is used in manufacturing polylactic acid (Bozell and Petersen 2010), which has recently been studied with great interest as a biodegradable polymer that can be used in the production of food packaging, plastic utensils, and plastic sheeting used for parts of computers, thereby replacing products made from petroleum (Ohara 2003; Coelho et al. 2011a, b). L(+) Lactic acid is used in the food industry to preserve human foodstuffs and is also utilized in the pharmaceutical and cosmetic industries (Li et al. 2004; Naveena et al. 2005; Wee et al. 2006a, b).

Some microorganisms can produce optically pure L(+) or D(−) lactic acid isomers via fermentation, while racemic DL-lactic acid is produced via chemical synthesis (Hofvendahl and Hahn-Hägerdal 2000; Lima et al. 2010). In the literature, there are more studies involving L (+) lactic acid than there are for D(−) lactic acid production, and additionally, the production yield of D(−) lactic acid is lower than that of L(+) lactic acid (Wang et al. 2011).

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Design experiments have been frequently used for optimizing media to improve lactic acid production (Chauhan et al. 2006; Mel et al. 2008). Methods such as factorial design and response surface methodology provide powerful and efficient ways to optimize cultivation and procedures using a reduced number of experiments (Mandenius and Brundin 2008).

The quality of the lactic acid from fermentation media is closely related to recovery and purification methodologies (González et al. 2006). Typically, lactic acid for use in polymers, pharmaceuticals, or food derivatives requires further purification steps; however, the inclusion of these steps is responsible for the high cost of lactic acid obtained by fermentation (Timbuntam et al. 2008). Thus, to reduce costs and solve environmental problems, many studies of the recovery and purification of lactic acid from fermentation media have been conducted using different separation techniques (Marinova et al. 2004; González et al. 2006; Timbuntam et al. 2008).

The present study reports on the highly efficient production of D(−) lactic acid by *S. nakayamae* using peanut flour as a nitrogen source to reduce the production cost. Fermentation conditions were optimized through experimental design. Additionally, a new methodology for the recovery and purification of D(−) lactic acid from the fermentation medium by filtration through activated carbon and Celite, followed by cation exchange chromatography is presented.

Materials and Methods

Bacteria strain and culture conditions

No specific permissions were required for these locations because this microorganism was collected within the university. *S. nakayamae*, a homofermentative lactic acid bacterium that produces D(−) lactic acid with high optical purity, was used in this study. This microorganism was isolated from the rhizosphere of *Desmodium adscendens* collected in the state of São Paulo (Brazil). The strain was stored in GYP medium with 20 % (by volume) glycerol at −80 °C. GYP medium is composed of 2 % glucose, 1 % yeast extract, 1 % peptone, 1 % sodium acetate, and 0.5 % (v/v) salt solution, which was comprised of 4 % $MgSO_4 \cdot 7H_2O$, 0.16 % $MnSO_4.4H_2O$, 0.2 % $FeSO_4.7H_2O$ and 0.2 % NaCl; pH 7.0.

Inoculum and shake-flask fermentation

Inoculum was prepared from activated culture grown on GYP medium incubated at 35 ± 1 °C under stationary conditions until the OD_{600} reached 2.5 (approximately 18–20 h). Experiments were performed in 125-mL flasks containing 18 mL of GYP medium at pH 7.0 and inoculated with 2 mL of the seed culture (10 % inoculum). To avoid a pH decrease because of lactic acid production, 50 g/L of $CaCO_3$ was added to each flask. The temperature was kept at 35 °C. The cultures were incubated at different conditions, as described below. All experiments were carried out in duplicate to verify their reproducibility, and the results are expressed as mean values. The fermentation samples were centrifuged at 7000 × g for 15 minutes. The resulting supernatant was filtered through a 0.22-μm membrane and was then used to determine the concentration of residual sugar and D(−) lactic acid .

Lactic acid production on different carbon and nitrogen sources

Lactic acid production was studied with different substrates as carbon and nitrogen sources based in GYP medium, with incubation for 48 hours at 35 °C under shaker conditions at 150 rpm.

After the sources that induced the highest lactic acid levels were selected, several concentrations of the carbon and nitrogen sources were evaluated using the experimental designs described below.

Experimental designs

Screening of significant nutritional components of lactic acid production using the Plackett-Burman Experimental Design (PBED)

The PBED was employed for screening and identifying the significance of eight parameters for D(−) lactic acid production. The GYP medium was modified to evaluate the influence of different nutritional components on lactic acid production. Each factor was tested at two levels (coded): a high level (+1) and a low level (−1) (Table 1), and four central points were screened

Table 1 Variables and their levels in lactic acid production under a PBED

Variable	Code	Levels		
		Low (−1)	0	High (+1)
Tween80 (ml/L)	X_1	0	0.5	1
$MnSO_4$ (g/L)	X_2	0	0.05	0.1
$FeSO_4$ (g/L)	X_3	0	0.05	0.1
NaCl (g/L)	X_4	0	0.05	0.1
$MgSO_4$ (g/L)	X_5	0	1	2
Peanut flour (g/L)	X_6	40	60	80
Sodium acetate (g/L)	X_7	0	5	10
Commercial sucrose (g/L)	X_8	80	115	150

Samples were incubated at a temperature of 35 ± 1 °C for 48 hours at 100 rpm. A Fractionated Factorial Design (FFD) was performed according the variables that significantly influenced the production of lactic acid

by running 16 experiments. The factors that were significant at the 5 % level ($P < 0.05$) from the regression analysis were considered to have a high impact on lactic acid production.

Fractional factorial designs

Five variables were studied in a 2^{5-1} FFD to estimate their effect on lactic acid production. A total of 19 experiments were carried out, with 16 experiments related to the design structure and three repetitions at the central point. The two extreme levels are denoted by minus one (lower level) and plus one (higher level), as shown in Table 2.

A new approach of GYP salts was evaluated, and it was added to the solution at certain concentrations. Once peanut flour was established as a nitrogen source, the influence of B vitamins on the production of lactic acid was examined. The fermentation was carried out under shaker conditions (100 rpm) at 35 °C for 48 hours. After incubation, the cultures were processed and quantitatively assayed for lactic acid and residual sugar production.

Response Surface Methodology (RSM)

The RSM was applied to evaluate the interaction of variables and then used to find the optimum concentration of the medium components that greatly affect D(−) lactic acid production (Table 3). To study the influence of commercial sucrose, peanut flour, and Tween 80 on the production of lactic acid by *S. nakayamae*, an RSM was used, with four replicates at the center, totaling 18 experiments.

The flasks were incubated for 72 hours at 35 °C under shaker conditions at 100 rpm. Lactic acid and residual sugar were quantified.

Experimental validation

To validate the optimum values selected by the response surface analysis, two conditions were tested. The concentrations utilized were: 110.00 g/L of commercial sucrose, 150.40 of peanut flour, and two different concentrations of Tween 80, 1.00 ml/L and 0.16 ml/L.

The fermentation was carried out under shaker conditions (100 rpm) at 35 °C for 72 hours. Each condition was tested five times, and the results are expressed as the mean values.

Table 2 Design matrix for the fractional 2^{5-1} factorial experiments, used to study the influence of 5 factors on *S. nakayamae* D(−) lactic acid production

Variable	Code	Levels		
		Low (−1)	0	High (+1)
Commercial sucrose (g/L)	X_1	80	100	120
Peanut flour (g/L)	X_2	60	80	100
Tween 80 (ml/L)	X_3	0.5	1	1.5
GYP Salts solution (g/L)	X_4	0	2.5	5
Vitamins (ml/L)	X_5	0	5	10

Table 3 Variables and their levels of lactic acid production under an RSM

Variable		Levels				
		- 1,68	−1	0	+1	+1,68
Commercial sucrose (g/L)	X_1	76,40	90	110	130	143,60
Peanut Flour (g/L)	X_2	49,60	70	100	130	150,40
Tween 80 (ml/L)	X_3	0,16	0,5	1,00	1,5	1,84

Purification and recovery of D(−) lactic acid

The fermentation medium was centrifuged at 7000 × *g* at 20 °C for 10 min, and the pH was adjusted to 5.0. Then, 60 ml of the medium (88.27 g/L D(−) lactic acid and 13.50 g/L of sugar) was vacuum filtered through a sintered plate funnel (3.50 cm diameter and 5.00 cm high) containing 1 cm of activated carbon and 1 cm of Celite. The filtrate was filtered through activated carbon and Celite twice, then passed through a cation exchange column (Ambertile IRA 120 hydrogen forms), and filtered through a 0.22-μm membrane. Then, the final solution was used to determine the lactic acid, sugar, and protein concentrations. The total protein concentration was determined by the Lowry method (Peterson 1977).

The lactic acid recovery yield was calculated as follow: lactic acid recovery yield (%) = (final mass (g) / initial mass (g)) × 100.

The sugar removal was calculated using this formula: sugar removal (%) = ((initial mass (g) - final mass (g))/ initial mass (g)) × 100.

Analytical techniques

The concentrations of sucrose, lactose, and lactic acid were determined by high-performance liquid chromatography (HPLC) using a column Rezex ROA 300 mm × 7.8 m (Phenomenex, USA) and a differential refracting index detector (Shimadzu Ultra Fast Liquid Chromatograph, Japan). The mobile phase (0.005 M H_2SO_4) was fed at a flow rate of 0.6 mL/min, and the temperature was kept at 65 °C.

In the end of the optimization process of D(−) lactic acid production, the optical purity of lactic acid produced was determined by HPLC using a Chirex 3126 phenomenex (150 × 4.6 mm) column with 1 mM of CuSO4 as the mobile phase at 1 mL/min (26 °C).

Statistical analysis

The experimental design and the results analysis in all experimental designs were determined using STATISTICA 7 (StatSoft, Tulsa, USA).

Results and discussion

Influence of carbon sources

D(−) Lactic acid production by *S. nakayamae* under the influence of four inexpensive carbon sources was studied (Table 4).

The carbon source that induced the highest lactic acid production was commercial sucrose, producing 59.50 g/L of lactic acid. *S. nakayamae* was also able to produce lactic acid in the presence of sugarcane juice, yielding 53.80 g/L of lactic acid. In the presence of whey and molasses, low levels of lactic acid were produced compared to those of the cultures grown with commercial sucrose and sugarcane juice. It may be that growing the cultures with molasses produced low levels of lactic acid because of the heavy metals (iron, zinc, copper, manganese) found in this substrate, which may inhibit cell growth and inactivate the enzymes involved in final product conversion. However, in previous studies, it was shown that L(+) lactic acid production by *Enterococcus faecalis* from molasses reached a maximum concentration of 134.9 g/L, with a maximum productivity of 4.3 g/Lh (Wee et al. 2006a, b). In the presence of whey, the low level of D(−) lactic acid production should be caused by the inability of *S. nakayamae* to metabolize the lactose, which is the sugar present in this residue, as reported before by Sanders et al. 2003 when referring to the other species of the genus *Sporolactobacillus*. In contrast, in a previous study, it was reported that 52.5 g/L of lactic acid was produced from whey by *Lactobacillus helveticus* (Ghaly et al. 2004).

Among the sources tested, commercial sucrose was selected. Although sugarcane juice was also capable of inducing lactic acid production, this substrate is less interesting in regards to further purification.

Influence of nitrogen sources

Different sources of nitrogen were also studied: corn-steep liquor, Proflo, peanut flour, soybean flour, and urea. The effects of each of these nitrogen sources on lactic acid production are shown in Table 5.

The nitrogen source that induced the highest lactic acid production was peanut flour, corresponding to 57.00 g/L of lactic acid. Moreover, Proflo, induced the production of 52.2 g/L of D(−) lactic acid, and a yield of 0.70 g of lactic acid/g of Proflo. Thus, it was the second most efficient nitrogen source in the production of lactic acid by *S. nakayamae*. This source presents some vitamins on composition, which possibly have a role on this fermentation. Peanut flour and soybean flour have several minerals in their composition (calcium, iron, mMagnesium, manganese, and sodium), which can act as enzyme cofactors. Similar to this study, it has been previously shown that *Bacillus* sp. and *Sporolactobacillus* sp. CASD produce high levels of L(+) and D(−) lactic acid, respectively, using peanut flour as their sole nitrogen source (Meng et al. 2012; Wang et al. 2011).

Moreover, in another previous study, it was reported that *Lactobacillus* sp. produced 41.42 g/L of D(−) lactic acid using corn step liquor and yeast autolysate (Lima et al. 2009). In the present study, when only corn step liquor was used as a nitrogen source, the lactic acid production was not as efficient, yielding 13.26 g/L of D(−) lactic acid . Furthermore, it left a high concentration of residual sugar (70.00 g/L).

Because the nitrogen source accounts for approximately 38 % of the total process cost during lactic acid production (Altaf et al. 2007), the use of wastes and inexpensive nitrogen sources in fermentation medium would reduce these costs.

Experimental designs

Plackett-Burman Experimental Design

The PBED is an efficient screening method for identifying the active factors of an experiment using as few experimental runs as possible.

Table 4 Influence of carbon sources on *S. nakayamae* lactic acid production, productivity, and yield

Carbon sources	Carbohydrates	D(−) Lactic acid Production (g/L)	Productivity (g/Lh)	Yield Yp/s (g/g)	Residual sugar (g/L)
Molasses	46,9 (g/100 mL)[a]	1.06	0.02	0.12	91.40
Commercial sucrose	100 (g/100 g)[a]	59.50	1.23	0.69	14.80
Sugarcane juice	19.95 (g/100 mL)[a]	53.80	1,12	0.66	19.20
Whey	76.59 (g/100 g)[b]	1.26	0.02	0.11	88.57

Culture conditions: GYP medium with 100 g/L of carbon source, 5 % of $CaCO_3$, at 35 °C under shaker conditions at 150 rpm, for 48 h, with an initial pH of 7.0. Type of carbohydrate: a)sucrose; b) lactose

Table 5 Influence of nitrogen sources on *S. nakayamae* lactic acid production, productivity, and yield

Nitrogen sources	N %	D(−) Lactic acid Production (g/L)	Productivity (g/Lh)	Yield Yp/s (g/g)	Residual sugar (g/L)
Proflo	11.15	52.20	1.09	0.71	26.43
Corn step liquor	4,69	13.26	0.28	0.44	70.00
Urea	46.62	38.40	0.80	0.78	51.00
Soybean flour	6.40	41.28	0.86	0.60	30.81
Peanut flour	7.63	57.00	1.19	0.72	21.2

Culture conditions: GYP medium with 100 g/L of commercial sucrose, 5 % of $CaCO_3$, at 35 °C under shaker conditions at 150 rpm, for 48 h, at an initial of pH 7.0. The nitrogen content of all media was 15.25 % ,

The matrix (coded values) of the 16 experiments with eight variables and the respective results are shown in Table 6. After 48 hours of fermentations, the maximum level of lactic acid production was 58.90 g/L (run 7).

The Pareto graph implied that the variables $MgSO_4$, peanut flour, Tween 80, and commercial sucrose influenced the fermentation process significantly at a 95 % confidence level. $MgSO_4$ and commercial sucrose had negative coefficients, while peanut flour and Tween 80 showed positive coefficients (Fig. 1a).

Kitouni and Oulmi (2013) using PBED reported that the use of manganese, magnesium, and iron sulfates as sources of oligoelements appears to have no significant effect on the lactic acid production by *Lactobacillus bulgaricus* attc 11842 grown on whey.

Once the peanut flour presents high lipid concentration in the composition (51.77 g/100 g), the surfactant property of Tween 80 can interact with lipids, improving the solubility of the nutrients found in peanut flour. Tween 80 may assist in nutrient absorption by microorganisms, as well as product liberation by facilitating transport via the cell membrane of the microorganism. Conversely, Coelho et al. 2011a, b reported that Tween 80 had a significant positive effect on lactic acid production by *Lactobacillus plantarum* LMISM6.

The negative effect of commercial sucrose is probably related to the high concentration used, which could inhibit the lactic acid production. Therefore, the components selected for the next experiment were peanut flour, commercial sucrose, and Tween 80.

Several studies reported the use of PBED successfully employed to screen the significance components in culture media, in order to improve the lactic acid production (Wang et al. 2015; Qin et al. 2009; Chauhan et al. 2007). Gowdhaman et al. (2012) using PBED was able to select three variables

Table 6 PBED with coded values and experimental results for lactic acid production, productivity, yield, and residual sugar concentration

Run	Independent variables								Lactic acid g/L	Productivity g/L/h	Yield Y p/s g/g	Residual sugar g/L
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8				
1	1	−1	1	−1	−1	−1	1	1	52.44	1.09	0.80	84.35
2	1	1	−1	1	−1	−1	−1	1	44.40	0.92	0.69	86.02
3	−1	1	1	−1	1	−1	−1	−1	30.43	0.63	0.99	49.15
4	1	−1	1	1	−1	1	−1	−1	55.85	1.16	0.98	22.92
5	1	1	−1	1	1	−1	1	−1	51.05	1.06	0.97	27.63
6	1	1	1	−1	1	1	−1	1	45.78	0.95	0.78	91.09
7	−1	1	1	1	−1	1	1	−1	58.90	1.22	0.99	23.75
8	−1	−1	1	1	1	−1	1	1	15.64	0.32	0.76	133.72
9	−1	−1	−1	1	1	1	−1	1	40.96	0.85	0.77	96.73
10	1	−1	−1	−1	1	1	1	−1	54.73	1.14	0.98	24.42
11	−1	1	−1	−1	−1	1	1	1	48.70	1.01	0.40	98.93
12	−1	−1	−1	−1	−1	−1	−1	−1	51.73	1.08	0.95	25.42
13	0	0	0	0	0	0	0	0	51.19	1.07	0.96	61.61
14	0	0	0	0	0	0	0	0	46.38	0.97	0.76	53.70
15	0	0	0	0	0	0	0	0	50.25	1.05	0.78	50.60
16	0	0	0	0	0	0	0	0	51.59	1.07	0.94	59.97

X_1 = Tween80. X_2 = $MnSO_4$. X_3 = $FeSO_4$. X_4 = NaCl. X_5 = $MgSO_4$. X_6 = Peanut flour. X_7 = Sodium acetate. X_8 = Commercial sucrose. Culture conditions: 35 °C, initial pH of 7.0 neutralized by 5 % $CaCO_3$, 100 rpm of agitation, 10 % inoculum. Fermentation was carried out for 48 hours

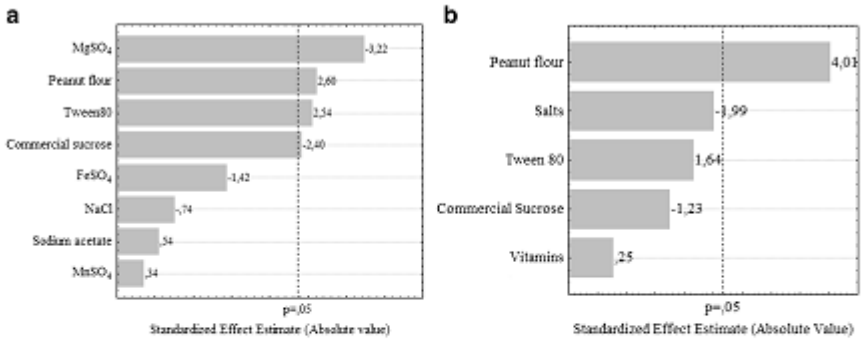

Fig. 1 Pareto graph for D(−) lactic acid production. a) PBED; b) FFD

namely substrate concentration, moisture content, and yeast concentration that have statistically significant effect on lactic acid yield by *Lactobacillus plantarum* MTCC 6161 in solid-state fermentation. Chauhan et al. 2006, studying optimization of lactic acid production using date juice, reported that among the fifteen variables (date juice, peptone, beef extract, yeast extract, K2HPO4, KH2PO4, MgSO4 · 7H2O, MnSO4 · H2O, sodium acetate, sodium sulfate, tri-sodium citrate, sodium succinate, tween-80, F,eSO4 and NaCl), significant components were screened out using PBED . This experiment suggested four components, date juice, peptone, K2HPO4, and sodium acetate, as having significant effects on lactic acid production by *Lactobacillus* sp. KCP01.

Fractional factorial designs (FFD)

In this study, a 2^{5-2} FFD was used to determine the significant factors of five media components. The 19 runs and the results of the response FFD are presented in Table 7.

Table 7 Fractional factorial design with coded and experimental results for lactic acid production, productivity, yield, and residual sugar concentration

Run	Independent variables					Lactic acid	Productivity	Yield Yp/s	Residual sugar
	X_1	X_2	X_3	X_4	X_5	g/L	g/Lh	g/g	g/L
1	−1	−1	−1	−1	1	48.49	1.01	1.20	39.70
2	1	−1	−1	−1	−1	41.76	0.87	0.48	33.15
3	−1	1	−1	−1	−1	47.82	1.00	0.67	8.70
4	1	1	−1	−1	1	46.46	0.97	0.70	53.74
5	−1	−1	1	−1	−1	46.24	0.96	0.62	5.35
6	1	−1	1	−1	1	44.35	0.92	0.85	67.86
7	−1	1	1	−1	1	49.02	1.02	0.97	29.21
8	1	1	1	−1	−1	51.92	1.08	0.87	60.04
9	−1	−1	−1	1	−1	42.79	0.89	0.99	36.85
10	1	−1	−1	1	1	43.05	0.90	0.80	66.22
11	−1	1	−1	1	1	46.87	0.98	1.04	35.08
12	1	1	−1	1	−1	44.01	0.92	0.83	66.79
13	−1	−1	1	1	1	42.16	0.88	1.01	38.08
14	1	−1	1	1	−1	42.79	0.89	0.80	66.63
15	−1	1	1	1	−1	49.59	1.03	0.98	29.53
16	1	1	1	1	1	48.59	1.01	0.98	70.22
17	0	0	0	0	0	43.15	0.90	0.85	49.41
18	0	0	0	0	0	44.205	0.92	0.89	50.13
19	0	0	0	0	0	44.465	0.93	0.86	48.52

X_1 = Commercial sucrose X_2 = Peanut flour. X_3 = Tween 80. X_4 = Salt solution. X_5 = Vitamins. Culture conditions: 35 °C, initial pH 7.0 neutralized by 5 % $CaCO_3$, 100 rpm of agitation, 10 % inoculum. Fermentation was carried out for 48 hours

Table 8 RSM design with coded values and experimental results for lactic acid production, productivity, yield, and residual sugar concentration

Run	Independent variables			Lactic acid	Productivity	Yield Y p/s	Residual sugar
	X_1	X_2	X_3	g/L	g/Lh	g/g	g/L
1	−1	−1	−1	78.10	1.08	0.88	15.20
2	1	−1	−1	85.48	1.19	0.89	54.97
3	−1	1	−1	81.67	1.13	0.79	9.95
4	1	1	−1	99.46	1.38	0.85	22.85
5	−1	−1	1	83.77	1.16	0.92	9.89
6	1	−1	1	96.18	1.34	0.86	28.32
7	−1	1	1	78.81	1.09	0.91	6.80
8	1	1	1	101.99	1.42	0.91	26.13
9	−1,68	0	0	82.40	1.14	0.95	5.39
10	1,68	0	0	88.68	1.23	0.79	41.50
11	0	−1,68	0	88.53	1.23	0.91	23.80
12	0	1,68	0	99.99	1.39	0.86	10.24
13	0	0	−1,68	93.47	1.30	0.84	16.24
14	0	0	1,68	93.11	1.29	0.84	17.92
15	0	0	0	93.53	1.30	0.88	20.88
16	0	0	0	91.60	1.27	0.84	17.80
17	0	0	0	88.80	1.23	0.85	22.66
18	0	0	0	92.02	1.28	0.85	19.12

X1 = Commercial sucrose, X2 = Peanut flour, X3 Tween 80. Culture conditions: 35 °C, Initial pH 7.0 neutralized by 5 % $CaCO_3$, 100 rpm of agitation, 10 % inoculum. Fermentation was carried out for 72 hours

The highest lactic acid concentration was identified on run 8 (52.92 g/L). This run contained the highest concentrations of carbon and nitrogen sources and contained no salts or vitamins; however, the level of residual sugar found in this experiment was high (60.04 g/L).

After 48 hours of fermentation, the peanut flour had a positive and significant effect on D(−) lactic acid production (Fig. 1b).

The commercial sucrose and salts, despite being presented as not significantly influential, had negative effects on lactic acid production. This negative effect may be associated with the amount of carbohydrates and mineral salts that already exist in peanut flour, causing a high concentration of these components on the culture media. As previously mentioned, the negative effect of commercial sucrose is related to product inhibition mediated by high substrate concentration. Different

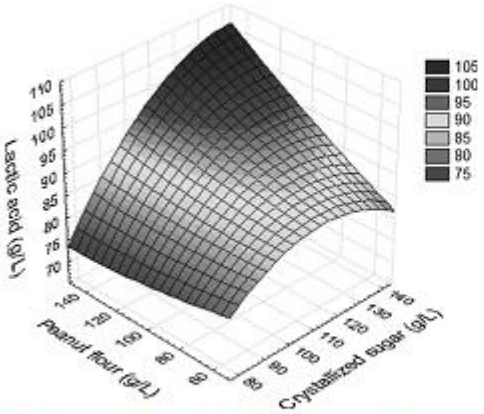

Fig. 2 Response surface for lactic acid production by *S. nakayamae* showing the interaction between peanut flour and commercial sucrose

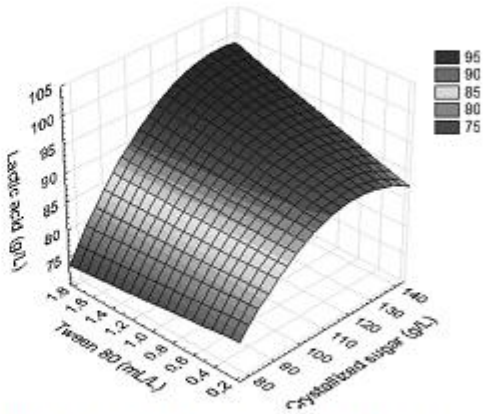

Fig. 3 Response surface for lactic acid production by *S. nakayamae* showing the interaction between Tween 80 and commercial sucrose

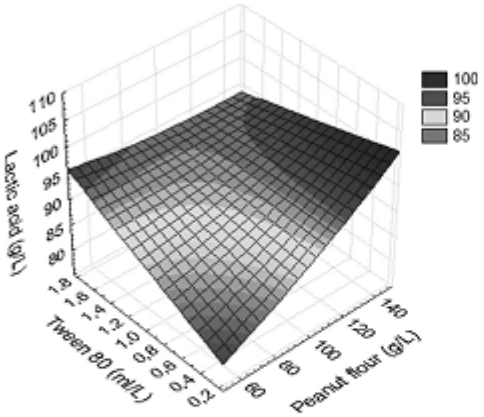

Fig. 4 Response surface for lactic acid production by *S. nakayamae* showing the interaction between Tween 80 and peanut flour

results were reported by Vasala et al. (2005), who showed that *Lactobacillus salivarius* ssp. is an ideal organism for lactic acid fermentation in high-salt high-lactose conditions. Additionally, the vitamins and Tween 80 did not significantly influence lactic acid production; nevertheless, it was verified that when the highest concentration of Tween 80 was used, there was higher lactic acid production. However, previous studies reported a positive influence of B vitamins on lactic acid production (Yoo et al. 1997; Altaf et al. 2007).

Considering these results, Tween 80, commercial sucrose, and peanut flour were selected for use in the RSM.

Response Surface Methodology (RSM)

Three factors, commercial sucrose, peanut flour, and Tween 80, were distributed in three levels, and two axial points and four central points were evaluated. The responses measured were D(−) lactic acid concentration, productivity, yield, and residual sugar after 72 h of fermentation (Table 8). Among the concentration of nutrients evaluated, 130 g/L of commercial sucrose, 130 g/L of peanut flour, and 1.5 ml/L of Tween 80 (run 8) achieved the highest D(−) lactic acid production (101.99 g/L) and productivity (1.42 g/L h), although there was a high concentration of residual sugar (26.13 g/L).

The results obtained were fitted to multiple regression analysis to yield the following regression equation:

$$Y = 91.62 + 5.22X_1 + 2.75X_2 + 1.12X_3 - 2.71X_{1^2} + 2.64X_1X_2 + 1.30X_1X_3 + 0.37X_{2^2} - 2.08X_2X_3 + 0.02X_{3^2}$$

in which Y is the predicted response (D(−) lactic acid production) and X1, X2, and X3 are the coded values of the test variables for commercial sucrose, peanut flour, and Tween 80, respectively.

An ANOVA of the quadratic regression model demonstrated that the model is significant, as determined by Fisher's test (Fcalc (3.81) > Ft (3.39)). It was also determined that the model has a very low probability value ((Pmodel > F) =0.03). The fit of the model was checked by the coefficient of determination (R^2) and multiple correlation coefficients (R). The R^2 value (0.81) for the regression equation indicated that the sample variation for lactic acid of 81 % was attributed to the independent variables. In this case, the value of the coefficient of determination indicated that only 19 % of the total variations were not explained by the model. The high R value (0.90) demonstrated a strong agreement between observed and predicted values. Among the model terms, X1 was significant with a probability of 95 %.

The response for the regression equation was plotted in Figs. 2, 3, and 4. The following graphs show the interaction of the variables and the optimum levels of each variable for D(−) lactic acid production.

Based upon the response surfaces and the values presented in Table 8, run 12 was considered the most appropriate combination for D(−) lactic acid production. This run consisted of 110 g/L of commercial sucrose, 150.40 g/L of peanut flour, and 1 mL/L of Tween 80, producing 99.99 g/L of D(−) lactic acid, with a 1.39 g/Lh rate of productivity and a low concentration of residual sugar (10.24 g/L).

When the highest levels of commercial sucrose and peanut flour were used, it was verified that this condition produced high levels of lactic acid; however, the amount of residual sugar produced was substantial. The same results were verified on

Table 9 Purification parameters of the fermentation media

Parameters	Lactic acid	Sugar	Protein
[Initial solution] (g/L)*	88.27	13.50	13.54
Initial mass (g)	5.296	0.810	0.812
[Final solution] (g/L)**	16.85	0.06	0.00
Final mass (g)	4.212	0.015	0.000
Index	Recovery Yield 79 %	sugar removal 98 %	protein removal 100 %

* Initial volume 60 mL; ** Final volume 250 mL

commercial sucrose and Tween 80 interaction. The response surface between peanut flour and Tween 80 showed that intermediate concentrations of this components did not produce a satisfactory amount of D(−) lactic acid . Additionally, it was found that low Tween 80 concentrations combined with high peanut flour concentrations showed higher levels of D(−) lactic acid production.

Previous studies have reported that RSM is an efficient method for the determination of optimized culture conditions for the production of lactic acid (Lima et al. 2011; Coelho et al. 2011a, b). Chaisu et al. (2014) reported, using RSM, the best percentage of inoculum (8.02 %) and carbon source (3.82 % of molasses) to lactic acid production (38.33 %) by *Lactobacillus casei* M-15 within 24 hr at 37 °C. Bustos et al. 2004 demonstrated the optimization of culture media applying RSM using *Lactobacillus coryniformis*. The models predicted a maximum D(−) lactic acid concentration (58.9 g/L) at 96 h using 5 g corn steep liquor/L, 3.6 g Yiest Extract/L, and 10 g Peptone/L. In turn, Lima et al. (2010) through RSM verified that the best results for lactic acid production (52.37 g/L) were obtained with 59.64 g/L of lactose, 14.55 g/L of corn steep liquor, and 5.65 g/L of (NH4)2SO4, at 39.6 °C by *Lactobacillus* sp. LMI8.

Experimental validation

A low concentration of Tween 80 (0.16 mL/L) combined with 110 g/L of commercial sucrose and 150.40 g/L of peanut flour induced a higher D(−) lactic acid production (112.93 g/L), productivity (1.57 g/L.h), and yield (0.98 g/g) with a reduced residual sucrose concentration (9.6 g/L). Compared with conditions utilizing a higher concentration of Tween 80 (1.00 mL/L), low levels of D(−) lactic acid (104,38 g/L), productivity (1,45 g/L.h) and yield (0,96 g/g) were presented.

In order to verify the optical purity of lactic acid produced, the optimized culture media was analyzed by HPLC using a chiral column. *S. nakayamae* produced 98.75 % of D(−) lactic acid and 1.24 % of L(+) lactic acid, thus it is considered a homofermentative microorganism.

Purification and recovery of D(−) lactic acid

The centrifuged fermentation medium at pH 5.0 was submitted to the purification process, which involved two steps: (1) double filtration on activated carbon and Celite, and (2) the transformation of calcium lactate into lactic acid via cation resin exchange. The purification and recovery assays were performed in duplicate, and the results are expressed as the mean values (Table 9).

D(−) lactic acid was efficiently recovered from fermentation medium (79 %). Additionally, protein and sugar were efficiently removed (100 % and 98 %, respectively). Previous studies have reported similar recovery yields using other purification techniques, such as solvent extraction, multiple-pass distillation, and micellar-enhanced ultrafiltration (Chen et al. 2012; Geanta et al. 2013). Furthermore, it was verified that there was a low ratio of sugar to lactic acid (0.36 %), which is essential to polymer synthesis for avoiding caramelization.

Conclusions

In this study, it was found that *S. nakayamae* is able to produce high levels of D(−) lactic acid using peanut flour and commercial sucrose as substrates. Experimental design methodology effectively determined the significant nutrients and optimum concentration of medium components. The purification methodology works with inexpensive and reusable reagents. In addition, the high performance of this method of D(−) lactic acid production utilizing cheap materials will be beneficial for bioplastic technologies. However, to be applied on a large scale in the lactic acid industries, additional well-established research efforts need to conducted.

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Material suplementar referente ao artigo I **"High D(−) lactic acid levels production by *Sporolactobacillus nakayamae* and an efficient purification"**

Supplementary material

Table 10.

Tabela 6. ANOVA with estimated regression coefficients for D(-) lactic acid production

ANOVA with estimated regression coefficients for D(-) lactic acid production

Source	Coefficient	Sum of squares	Degree of freedom	Mean square	F ratio	P
X1	5.22	372.558	1	372.558	18.114	0.002*
X2	2.75	103.924	1	103.924	5.053	0.054
X3	1.12	17.421	1	17.421	0.847	0.384
X1X2	2.64	56.021	1	56.021	2.723	0.137
X1X3	1.30	13.572	1	13.572	0.659	0.440
X2x3	-2.08	34.861	1	34.861	1.695	0.229
X1X1	-2.71	93.133	1	93.133	4.528	0.066
X2X2	0.37	1.735	1	1.735	0.084	0.778
X3X3	0.02	0.008	1	0.008	0.000	0.984
Model	-	705.68	9	78.40	3.81	0.03
Error	-	164.53	8	20.56	-	-
Lack of fit	-	152.84	5	30.56	7.84	0.06
Pure error	-	11.68	3	3.89	-	-
Total	-	870.22	17	-	-	-

X1= Crystal sugar; X2 = Peanut flour; X3=Tween 80. *Statistically significant at a 95% probability.

Composition of carbon and nitrogen sources

Molasses

Sucrose (46,9g/100mL), Ash (11,5 g/100g), Calcium (0,9 g/100mL), Phosphorous (0,2 g/100mL), Potassium (0,6 g/100mL), Magnesium (0,6 g/100mL), Sodium (0,3 g/100mL), Iron (1,3 mg/mL), nitrogen (0,37 g/mL), zinc (1,1 mg/mL).

Sugar cane juice

Proteins (0.26 g/100 mL), Lipids (0.10 g/100 mL), Carbohydrates (19.95 g/100 mL), Moisture (79.47 g/100 mL) Ash (0.33 g/100 mL), Soluble solids (19.35 ºBrix).

Commercial sucrose

Calories (400.00 kcal /100g), Protein (0.00g /100g), Total Carbohydrates (100 g /100g), Sodium (0.00 mg /100g), Total fat (0.00 g /100g).

Whey

Calories (369.63 kcal/100g), Protein (13.32 g /100g), Total Carbohydrates (76.59 g/100g), Sodium (1078.92 mg/100g), Fiber (0.00 g/100g), Total fat (0.00 g/100g).

Proflo

Nitrogen (9.66 g/100g), Protein (60.38 g/100g), Carbohydrates (23.55 g/100g), reducing sugars (1.18 g/100g), nonreducing sugars(1.32 g/100g), Fat (4.24 g/100g), Ash (6.83 g/100g), Fiber (3.24 g/100g), Moisture (1.69 g/100g), Free gossypol (0.043 g/100g). Vitamin ug/g: Carotene 0.11, Tacopherols 14.0, Ascorbic acid 25.11, Thiamine 4.36, Riboflavin 5.11, Niacin 83.9, Pantothenic acid 12.51, Choline 33.48, Pyridoxine 0.88, Biotin 0.78, Folic acid 1.50, Inositol 3640.

Peanut flour

Ash (2.05 g/100g), Total Fat (51.77 g/100g), Total Carbohydrates (17.26 g/100g), Calories (640 kcal/100g), Protein (26.24 g/100g), Nitrogen (4.56 g/100g), Calcium (36.67 mg /100g), Iron (1.50 mg/100g), Magnesium (187 mg /100g), Manganese (2.12 mg/100g), Sodium (0.73 mg/100g).

Soybean flour

Calories (233.1 kcal /100g), Protein (39.96 g/100g), Nitrogen (6.39 g /100g), Total Carbohydrates (19.98 g /100g), Sodium (19.98 mg/100g), Fiber (19.98 g /100g), Total fat (39.6 g/100g).

Corn Step liquor

Nitrogen (4.69 g/100g), Protein ((29.31 g/100g), Lactose (3g/100g), Calcium (0.14%), Cooper (1,5 mg/100g), Manganese (2.0 mg/100g), Iron (10 mg/100g), Magnesium (0.6%), Potassium (2.8%), Sodium (0.1%), Phosphorus (1.8%). Vitamin mg/kg:, Thiamine 3.0, Riboflavin 6.00, Niacin 80.00, Pantothenic acid 15.00, Choline 3500.00, Pyridoxine 9.00, Biotin 0.3, Inositol 6000.00.