UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA QUÍMICA
DEPARTAMENTO DE ENGENHARIA DE MATERIAIS E BIOPROCESSOS

RHELVIS DE CAMPOS OLIVEIRA

Principais parâmetros de processo para produção microbiana do ácido hialurônico de alta massa molar e a sua purificação em carvão ativado

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Principais parâmetros de processo para produção microbiana do ácido hialurônico de alta massa molar e a sua purificação em carvão ativado

Autor – Rhelvis de Campos Oliveira

Orientadora - Profa. Dra. Maria Helena Andrade Santana

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Este trabalho corresponde à versão final da tese defendida pelo aluno Rhelvis de Campos Oliveira, orientado pela Profa. Dra. Maria Helena Andrade Santana.

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Dra. Aline Mara Barbosa Pires

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RESUMO

Neste trabalho foram estudadas estratégias para a produção do ácido hialurônico (AH) de alta massa molar (MM) utilizando *Streptococcus zooepidemicus* ATCC 39920 e meio de cultura composto de glicose e peptona de soja (PS). A adsorção em carvão ativado foi também estudada para a purificação do AH. As estratégias exploradas para a produção do AH foram: 1) os efeitos da proporção de aminoácidos livres em relação aos aminoácidos totais em PSs, 2) da cardiolipina adicionada ao meio de cultura, e 3) do oxigênio dissolvido (O.D). Para a primeira estratégia os cultivos foram conduzidos em biorreator de 3,0 L com volume útil de 2,5 L, sem controle de pH. Os efeitos da cardiolipina foram estudados em frascos Erlenmeyer somente. Em ambos os casos foram utilizadas razões carbono/nitrogênio (C/N) de 10,8 ou glicose/nitrogênio (G/N) de 4,3. A terceira estratégia foi estudada em biorreator sem aeração e com aeração forçada, em regimes de O.D não controlado, controlado e misto. Os resultados da primeira estratégia demonstraram uma relação direta entre a proporção de aminoácidos livres/aminoácidos totais na PS e a MM média do AH produzido, que por sua vez foi inversamente proporcional à velocidade específica máxima de crescimento celular (μ_{max}). A suplementação do meio de cultura com cardiolipina a 30 mg/L aumentou em aproximadamente 33% a MM média do AH nos cultivos realizados com PS. Com o aumento do O.D, houve aumento do μ_{max} com tendência de redução da MM média do AH. O controle do O.D a 90% da saturação ao longo de todo o cultivo favoreceu a produção do AH, porém com baixo rendimento (0,14 g/g) em relação à biomassa ($Y_{AH/Biomassa}$). Com o aumento da concentração inicial de glicose (70 g/L), o regime misto (controle de O.D a 90% da saturação após 3h de cultivo) apresentou maior rendimento $Y_{AH/Biomassa}$ (0,24 g/g) em relação ao controle contínuo de O.D (0,09 g/g). A adsorção em carvão ativado a 2.0% (m/v), em pHs 6.0 e 8.0, produziu AH com purezas de 91 e 96% respectivamente, em relação à concentração de proteínas e peptídeos totais (PPT), porém com perdas da ordem de 61 e 37% respectivamente no processo. Os resultados obtidos permitem concluir que as três estratégias estudadas assim como a adsorção em carvão ativado são promissoras para a produção do AH de alta MM e elevado grau de pureza. Esses resultados são apresentados nesta tese na forma de 4 artigos científicos que tratam das estratégias estudadas para produção e purificação do AH. Adicionalmente apresenta-se o resumo de uma patente depositada (BR 10 2017 027243 5), que aborda a utilização da farinha de soja como uma fonte inovadora de nitrogênio para a produção do AH.

ABSTRACT

In this work, strategies for the production of hyaluronic acid (HA) with high molar mass (MM) using *Streptococcus zooepidemicus* ATCC 39920 and culture medium composed of soy peptone (SP) and glucose were studied. Activated carbon adsorption was also studied for HA purification. The strategies explored for HA production were: 1) the effects of free amino acids in relation to total amino acids proportion in SPs, 2) cardiolipin added to the culture medium, and 3) dissolved oxygen (D.O). For the first strategy the cultures were carried in a 3.0 L bioreactor with a working volume of 2.5 L. The effects of cardiolipin were studied in Erlenmeyer flasks only. In both cases, carbon/nitrogen (C/N) of 10.8 or glucose/nitrogen (G/N) of 4.3 ratios were used. The third strategy was studied in bioreactor without aeration and with forced aeration in uncontrolled, controlled and a mixed regime of D.O control. The results of the first strategy demonstrated a direct relationship between the proportion of free amino acids/total amino acids in SP and the average MM of the produced HA, which in turn was inversely proportional to the maximum specific growth rate (μ_{max}). The supplementation of the culture medium with 30 mg/L cardiolipin increased by approximately 33% the HA average MM in cultures made with SP. With the increase of D.O, μ_{max} was increased with tendency to reduce the HA average MM. The D.O control at 90% saturation throughout the cultivation favored HA production, but with low yield (0.14 g/g) in relation to biomass produced ($Y_{HA/biomass}$). With the increase of the initial glucose concentration (70 g/L), the mixed regime (D.O controlled at 90% saturation after 3 h of cultivation) presented higher $Y_{HA/Biomass}$ yield (0.24 g/g) compared to continuous D.O control at 90% saturation (0.09 g/g). Activated carbon adsorption at 2.0 % (w/v), in pH 6.0 and 8.0, produced HA with purity of 91 and 96% respectively, in relation to total protein and peptides concentration (TPP), with losses in the order of 61 and 37% respectively. The results obtained allow us to conclude that the three strategies studied, as well as the adsorption in activated carbon, are promising for the production of increased HA MM with high purity grade. These results are presented in this thesis in the form of 4 scientific articles dealing with the strategies for production and purification of HA. In addition, a summary of a deposited patent (BR 10 2017 027243 5) is presented, which discusses the use of soybean meal as an innovative source of nitrogen for HA production.

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NOMENCLATURA

ADP - Adenosina difosfato

AH - Ácido Hialurônico

ATCC - American Type Culture Collection

ATP - Adenosina trifosfato

BHI - Brain Heart Infusion

CoASH - Coenzima A

C/N – Razão carbono/nitrogênio

O.D – Oxigênio dissolvido

AL - Aminoácidos livres

AT - Aminoácidos totais

HAS - Hialuronato sintase

HPLC - High performance liquid chromatography

MM – Massa molar

NAD^+ - Nicotinamida adenina dinucleotídio oxidada

NADH - Nicotinamida adenina dinucleotídio reduzida

pH - Potencial hidrogeniônico

RNA - Ácido ribonucléico

RPM - Rotações por minuto

PPT - Proteínas e peptídeos totais

UDP - Uridina difosfato

UTP - Uridina trifosfato

μ_{max} - Velocidade específica máxima de crescimento (1/h)

$Y_{Biomass/Glucose}$ – Fator de rendimento (grama de biomassa produzida por grama de glicose consumida) (g/g)

$Y_{X/S}$ – Fator de rendimento (grama de biomassa produzida por grama de glicose consumida) (g/g)

$\mathbf{Y_{HA/Glucose}}$ – Fator de rendimento (grama de ácido hialurônico produzido por grama de glicose consumida) (g/g)

$\mathbf{Y_{P/S}}$ – Fator de rendimento (grama de ácido hialurônico produzido por grama de glicose consumida) (g/g)

$\mathbf{Y_{HA/Biomass}}$ – Fator de rendimento (grama de ácido hialurônico produzido por grama de biomassa produzida) (g/g)

$\mathbf{Y_{P/X}}$ – Fator de rendimento (grama de ácido hialurônico produzido por grama de glicose consumida) (g/g)

$\mathbf{Y_{Acetate/Glucose}}$ – Fator de rendimento (grama de acetato produzido por grama de glicose consumida) (g/g)

$\mathbf{Y_{Lactate/Glucose}}$ – Fator de rendimento (grama de lactato produzido por grama de glicose consumida) (g/g)

SUMÁRIO

Introdução

O ácido hialurônico (AH) é um biopolímero de cadeia linear composto por repetitivas unidades dissacarídicas de ácido D-glicurônico (GlcUA) e N-acetil-glicosamina (GlcNAc) (Scott *et al.*, 1991). Possui amplas aplicações na medicina, farmacologia e cosmetologia, graças às características físico-químicas e biológicas que conferem à molécula elevada retenção de água, viscoelasticidade e biocompatibilidade. Em humanos, o AH é encontrado no cordão umbilical, humor vítreo e fluido sinovial, auxiliando na lubrificação e proteção de tendões e articulações (Meyer *et al*., 1939).

O AH foi descoberto em 1934 por Karl Meyer e John Palmer através de análises do humor vítreo bovino, o nome originou-se da junção dos termos hialóide (vítreo) e ácido urônico (Meyer e Palmer, 1934). Atualmente o AH comercial é produzido por via extração animal e fermentativa, a via fermentativa reduziria os riscos provocados pela presença de proteínas, ácidos nucleicos e vírus no AH extraído de fonte animal. Como vantagem existe também a possibilidade de otimizar o rendimento e a qualidade do produto através do controle das condições de produção e purificação do AH.

Segundo a revista Grand View Reasearch, o mercado global de AH foi avaliado em USD 7,2 bilhões em 2016, e espera-se que atinja o valor de USD 15,4 bilhões até 2025, por isso existe um forte interesse em otimizar o processo de produção do AH visando atingir elevados padrões de rendimento e qualidade a custos acessíveis. A aplicação do AH no mercado depende exclusivamente do seu grau de pureza e da sua massa molar (MM), estando ela entre 10^3 e 10^7 Da. O AH de elevada MM (MM > $1x10^6$ Da) atua como agente lubrificante no líquido sinovial, protegendo a cartilagem articular (Tamer, 2013). O AH de baixa e intermediária MM ($1x10^4$ - 10^6 Da) é capaz de estimular a produção de citocinas pró-inflamatórias, quimiocinas e fatores de crescimento (Cyphert *et al*., 2015).

O AH pode ser produzido através de microrganismos *Streptococcus equi.* sub. *equi* (Ellwood, *et al*., 1985)*, Streptococcus equi.* sub. *zooepidemicus* (Swann *et al*., 1990)*, Bacilus subtilis* (Widner *et al*., 2005) e *Escherichia coli* (Yu e Stephanopoulos, 2008). O produto obtido desses microrganismos possui as mesmas características do produto extraído de fonte animal ou presente em humanos, havendo variações em sua MM apenas. O AH é sintetizado no meio extracelular na forma de cápsulas, com a função de proteger o microrganismo contra o ataque de anticorpos. A via biosintética de produção do AH a partir da glicose é composta de duas rotas, glicose-6-fosfato e frutose-6-fosfato, dando origem respectivamente às

moléculas precursoras do AH (GlcUA e GlcNAc). Essas duas rotas são responsáveis também por fornecer os constituintes estruturais da parede celular bacteriana (peptideoglicanos e ácidos teicóicos), acirrando a competição por energia entre a biomassa e o AH produzido.

Streptococcus são bactérias nutricionalmente fastidiosas e requerem meio de cultura composto por fontes de carbono e nitrogênio para o seu crescimento e produção do AH. De acordo com a literatura, a fonte de carbono mais utilizada na produção do AH é a glicose e como fonte de nitrogênio encontramos o extrato de levedura. A fonte de nitrogênio complexa é composta por proteínas, peptídeos e aminoácidos, por isso questões relacionadas à factibilidade da purificação e redução de custos com meio de cultura poderiam ser solucionadas com a escolha da fonte de nitrogênio mais adequada.

A utilização de fontes vegetais de nitrogênio possibilita a redução dos riscos de reações imunogênicas provocadas por cadeias protéicas complexas provenientes de fontes comumente utilizadas como o extrato de levedura e o BHI (Brain and Heart Infusion). Nesse contexto, no presente trabalho foram estudados os efeitos da composição de aminoácidos livres e totais em peptonas de soja através de cultivos em biorreator, e da suplementação do meio de cultura com cardiolipina através de cultivos realizados em frascos Erlenmeyer. As razões carbono/nitrogênio (C/N) de 10,8 ou glicose/nitrogênio (G/N) de 4,3 foram utilizadas em todos os cultivos. Os resultados foram analisados através da cinética de produção de biomassa, AH, fatores de rendimento e MM do AH produzido.

Para o melhor entendimento do metabolismo do microrganismo nas condições nutricionais e ambientais presentes, a análise da distribuição de fluxos metabólicos é muito importante. Estudos recentes mostram que a produção e MM do AH são afetados pela disponibilidade de oxigênio no meio de cultura. Alguns autores relatam que o aumento dos níveis de oxigênio dissolvido (O.D) exerce influência positiva no rendimento e na MM do AH. *Streptococcus* são incapazes de utilizar a fosforilação oxidativa, entretanto na presença de oxigênio NADH é oxidado em NAD^+, e como resultado pode-se obter uma alteração na produção de metabólitos, a bactéria passaria a produzir mais acetato com concomitante produção de ATP, elevando o nível energético do sistema. Os efeitos do controle de O.D na produção e MM do AH também foram estudados, através de análises da cinética de crescimento celular, produção de AH e metabólitos (acetato e lactato), fatores de rendimento e MM do AH produzido.

A purificação do AH representa uma etapa de extrema importância pela sua vasta gama de aplicações. A concentração de proteínas e peptídeos totais (PPT) permitida no AH utilizado em aplicações médicas deve estar abaixo de 0,1%, por isso etapas físico químicas de

purificação são necessárias. No contexto de purificação foram realizados ensaios de adsorção com carvão ativado com o objetivo de estudar a remoção de PPT e recuperação do AH em condições distintas de concentração de carvão ativado e pH.

Os resultados obtidos e discussão serão apresentados na forma de artigos científicos com os seguintes títulos: “The amino acids profile soy-peptone as a determinant of the molar mass of hyaluronic acid synthesized by *Streptococcus zooepidemicus*” (Artigo 1), “Cardiolipin induces the production of high molar mass hyaluronic acid from *Streptococcus zooepidemicus* cultivation” (Artigo 2), “Oxygen control modulates microbial production and molar mass of hyaluronic acid synthesized by *Streptococcus zooepidemicus*” (Artigo 3) e “Effect of pH on adsorption with activated carbon for purification of hyaluronic acid” (Artigo 4).

OBJETIVO

O presente trabalho tem por objetivo estudar estratégias para a produção de ácido hialurônico de alta massa molar utilizando *Streptococcus zooepidemicus* ATCC 39920 e a sua purificação em carvão ativado.

As estratégias estudadas para atingir este objetivo foram:

1-Influência da composição e teor de aminoácidos em peptonas de soja comerciais.

2-Efeitos da suplementação do meio de cultura com cardiolipina.

3-Comportamento dos cultivos em função da concentração inicial de oxigênio dissolvido e do seu controle ao longo do processo.

4-Desempenho da purificação do ácido hialurônico em batelada por adsorção em carvão ativado.

REVISÃO BIBLIOGRÁFICA

1.1. BREVE HISTÓRICO

Em 1934, os cientistas Karl Meyer e John Palmer reportaram o isolamento de um glicosaminoglicano removido a partir do humor vítreo bovino. Apresentada como um polissacarídeo, essa molécula era composta por ácido urônico e um amino-açúcar, recebendo posteriormente o nome de ácido hialurônico (AH), junção do termo grego *hialóide* (vítreo) e *ácido urônico* (Meyer e Palmer, 1934). Em 1937, Kendall, Heidelberger e Dawson observaram uma semelhança entre o polissacarídeo extraído do microrganismo *Streptococcus,* grupo A hemolítico e a molécula recém descoberta, vislumbrando então a possibilidade da produção do AH por via microbiana, no entanto a presença de endotoxinas impedia a sua aplicação em humanos (Kendall *et al.*, 1937). Durante os anos de 1930 e 1950 o AH foi isolado a partir de diversas fontes, tais como, cordão umbilical humano (Meyer e Palmer, 1936), fluído sinovial humano (Meyer *et al.*, 1939) e crista de galo (Boas, 1949). A crista de galo se tornou a principal fonte de AH, por apresentar elevadas concentrações e massa molar (MM). Porém, procedimentos complexos de purificação eram necessários para a remoção dos contaminantes associados ao AH após a sua extração (Swann, 1968).

A semelhança entre a estrutura química do AH produzido por via microbiana e o AH encontrado em humanos viabilizou a sua produção em escala industrial em 1980, a partir do microrganismo *Streptococcus zooepidemicus,* contornando os riscos de contaminação viral do produto extraído de tecido animal (Akasaka *et al*., 1989).

1.2. ESTRUTURA, PROPRIEDADES E APLICAÇÕES

O AH é um mucopolissacarídeo com unidades repetitivas de um dissacarídeo composto por ácido D-glicurônico (GlcUA) e N-acetilglicosamina (GlcNAc) unidos por ligações glicosídicas β-1,3 e ligações glicosídicas β-1,4 entre as unidades monoméricas (Chong *et al.*, 2005).

A Figura (1.1) exibe a estrutura molecular do AH.

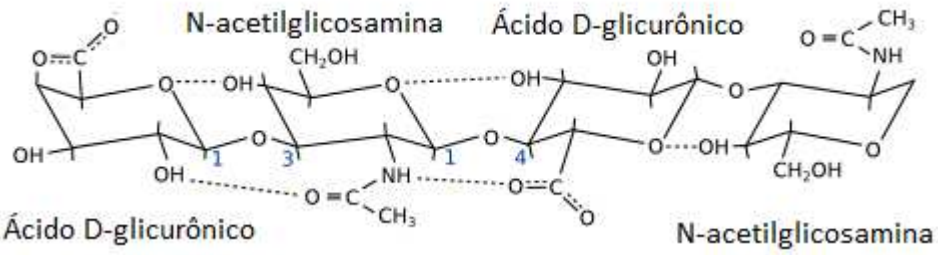

Figura 1.1- Estrutura molecular do ácido hialurônico (Scott *et al.*, 1991).

O AH cria uma estrutura de suporte no espaço extracelular e auxilia diretamente na proliferação, proteção, diferenciação celular e hidratação de tecidos (Hascall *et al.*, 1997). No fluido sinovial humano o AH atua como lubrificante das articulações e auxilia na absorção de impactos (Kim *et al.*, 1996). As funções do AH no corpo humano estão fortemente ligadas à sua massa molar (MM), caracterizada por apresentar uma ampla polidispersidade (entre 10^3 a 10^7 Da). Elevada MM (MM > $1x10^6$ Da) possui aplicações em preenchimentos de pele, imunossupressores e anti-inflamatórios (McBride e Bard, 1979; Feinberg e Beebe, 1983). Com base em dados recentes, o AH de elevada MM está emergindo como uma opção terapêutica viável a doenças inflamatórias das vias aéreas, devido às suas propriedades anti-inflamatórias. Dependendo da localização, o AH de elevada MM pode proteger células pulmonares da apoptose epitelial (Jiang *et al.*, 2005) e promover o processo fibrótico de cicatrização (Li *et al.*, 2011). O AH de média MM ($1x10^4$ ~ $1x10^6$ Da) possui propriedades cicatrizantes (Forrester e Balazs, 1980), enquanto que a MM reduzida ($4x10^2$ ~ $4x10^3$ Da) possui propriedades anti-apoptóticas estimulando o sistema imunológico, a expressão de citocinas pró-inflamatórias, quimiocinas e fatores de crescimento. Fragmentos de AH de baixa MM afetam o comportamento das células ligadas às proteínas receptoras, tais como CD44 e o RHAMM, sugerindo um biossensor do microambiente em torno da célula (Jiang *et al.*, 2005).

A estrutura química do AH é energeticamente muito estável, graças à configuração espacial de ambos os açúcares (GlcUA e GlcNAc). A estrutura do polímero é caracterizada por cadeias grandes e lineares nas quais o número de unidades repetidas de dissacarídeos, "n", pode chegar a 10.000. A combinação de estruturas químicas e poliméricas adicionadas às interações com a água conferem ao AH um esqueleto enrijecido em solução fisiológica, desta forma os átomos de hidrogênio axiais formam uma face relativamente hidrofóbica, enquanto as cadeias laterais equatoriais formam uma face hidrofílica (Hardingham, 2004). Essas faces são organizadas como fitas torcidas que ocupam um grande volume em solução. Esta é a razão para a grande capacidade de absorção do AH, aproximadamente 1000 vezes o seu peso em água (Cowman e Matsuoka, 2005).

Como a dissociação dos grupos carboxila do ácido glicurônico começa na faixa de pH 3-4, o AH se apresenta como um poliânion em pH fisiológico, com uma estrutura espiral expandida aleatória ocupando um grande domínio (Hascall e Laurent, 1997). Em elevadas concentrações de AH (1 mg/mL ou superior), esta configuração espacial permite a sobreposição de moléculas individuais (Fouissac *et al*., 1993). Em concentrações acima de 0,6 mg/mL e elevada MM (acima de $2x10^6$ Da), redes moleculares emaranhadas podem ser formadas através de interações inter ou intramoleculares, tais redes exibem propriedades diferentes das moléculas individuais, como propriedades elásticas e viscosas, dependendo do alinhamento de suas cadeias (Fouissac *et al*., 1993). O AH também forma malhas densas ao redor das células, através de receptores e sítios de ligação. Neste caso, o AH de alta MM produz um escudo mais eficiente na absorção de impactos e ação de radicais livres.

A relevância do conhecimento da estrutura química, polimérica e em solução do AH reside nos efeitos biológicos a que estão associados. O AH é um constituinte importante das matrizes extracelulares dos tecidos, no fluido articular sinovial o AH é o constituinte principal (1,4 - 3,6 mg/ml) (Kogan *et al*., 2007). As redes elásticas formadas pelo AH de alta MM podem distribuir forças de carga ou de cisalhamento dentro da rede que atuam como um absorvedor de impactos, evitando lesões e dor. Além disso, o AH também atua nas juntas como lubrificante, devido à sua estrutura altamente hidratada e propriedades reológicas.

O AH também desempenha papel estrutural essencial na cartilagem hialina, mesmo em concentrações reduzidas (~1 mg/g) em relação à agrecana (25-50 mg/g). Isso ocorre porque o AH retém moléculas de agrecana através de interações específicas proteína-AH em sua espinha dorsal hidrofóbica. A rede de AH também permite a livre difusão de água, pequenas moléculas e macromoléculas em menor extensão (Hascall e Laurent, 1997). Os fragmentos hidrofóbicos da molécula de AH podem formar complexos com mediadores

inflamatórios lipídicos, fator de ativação plaquetário e outras moléculas hidrofóbicas nocivas, reduzindo a inflamação dos tecidos articulares (Waddell *et al.*, 2007). O comportamento de dissociação dos grupos carboxílicos do AH também provocam alterações estruturais na molécula devido ao baixo pH transmitido pela inflamação. Em pH reduzido a molécula de AH pode alterar sua estrutura enovelada aleatória para uma estrutura de dupla hélice, reduzindo a viscoelasticidade do AH e a sua capacidade de reter moléculas hidrofóbicas (Cleland, 1968). Consequências semelhantes podem surgir da clivagem de cadeias de AH por espécies reativas de oxigênio, incluindo superóxido, óxido nítrico e outras espécies, geradas durante uma resposta inflamatória (Kalaci *et al.*, 2007).

A capacidade do AH de recobrir superfícies celulares pode fornecer proteção adicional aos condrócitos *in vivo* (Altman *et al*., 2015). Injeções ortobiológicas intra-articulares de AH têm sido uma opção de tratamento para a osteoartrite do joelho. Apesar do uso clínico extenso do AH como viscossuplemento para absorção de choques, lubrificação das juntas e alívio da dor, foi sugerido na literatura que estes não são os únicos efeitos do AH. Altman *et al*., (2015) realizaram uma extensa pesquisa bibliográfica (em torno de 90 artigos publicados), sobre os mecanismos de ação do AH em tratamentos de osteoartrite do joelho. Os resultados forneceram evidências de diversos mecanismos de ação do AH nas juntas e articulações. A condroproteção foi o mecanismo mais frequente, seguido da síntese de proteoglicanos e glicosaminoglicanos, das ações anti-inflamatórias, mecânicas, subcondrais e analgésicas.

O principal efeito da condroproteção é resultante da ligação do AH ao receptor CD44, a proteção reduz a apoptose dos condrócitos e aumenta a sua proliferação. O complexo AH-CD44 provoca inibição da expressão da interleucina (IL) 1β, levando a um declínio na produção de metaloproteinases da matriz que leva à degeneração da cartilagem (Julovi *et al*., 2004). A inibição da interleucina (IL) 1β também reduz o estresse oxidativo das espécies reativas de oxigênio (Peng *et al*., 2010). Chang *et al*., (2012) demonstrou que o AH de elevada MM exibe maior eficácia no surgimento da ligação AH-CD44. O AH de alta MM também contribui para a ação anti-inflamatória através da incorporação de citocinas inflamatórias e pré-inflamatórias em pH fisiológico (Lajeunesse *et al*., 2003).

Os efeitos mecânicos proporcionados pela viscoelasticidade e viscosidade do AH amortecem os impactos sofridos nas articulações e equilibram os efeitos do aumento do cisalhamento devido à degradação do AH nativo nos joelhos. Embora não seja um consenso, a maioria dos estudos demonstra os benefícios do AH de alta MM em relação ao AH de baixa MM (Chang *et al.*, 2012). Cyphert *et al*., (2015) apresentaram uma revisão com foco nas diferentes faixas de MM do AH e seus efeitos na sinalização e no comportamento das células

biológicas. Embora a MM do AH tenha um papel importante, os mecanismos envolvidos ainda são pouco compreendidos, consequentemente os efeitos biológicos do AH na homeostase tecidual e na resposta à lesão ainda não são claros.

Outro aspecto importante é a segurança do AH exógeno. Embora não haja evidência clínica, o AH produzido através de cultivo microbiano apresenta um perfil mais seguro. Eventos adversos como sinovite ou inflamações no local da aplicação, foram relatados com a utilização de AH derivado de aves (crista de galo). Atualmente, a maioria dos produtos comerciais disponíveis para tratamentos ortopédicos são de origem microbiana (Altman *et al.*, 2015). Novos produtos obtidos a partir da reticulação química do AH elevaram a estabilidade do biopolímero nas articulações. Pesquisas atuais em engenharia de tecidos utilizam scaffolds de AH para patologias espinhais, incluindo injeções intradiscais (Bauer *et al.*, 2013).

1.3. COMPORTAMENTO EM SOLUÇÃO

Em elevadas concentrações, a solução de AH apresenta consistência gelatinosa, com elevada viscoelasticidade e grau de hidratação (Kim *et al.*, 1996; Scott *et al.*, 1991). Em soluções fisiológicas diluídas de AH, os átomos axiais de hidrogênio formam uma face apolar hidrofóbica e as cadeias equatoriais formam uma face polar hidrofílica, criando um aspecto de fita retorcida na molécula (Hascall e Laurent, 1997). Pontes de hidrogênio entre as moléculas de água e os grupos carboxila e N-acetil conferem à molécula de AH elevada capacidade de retenção de água e dureza conformacional, limitando a sua flexibilidade (Lapcik *et al.*, 1998).

Em solução diluída, a conformação das cadeias de AH pode ser convertida de uma estrutura emaranhada (random coil) para uma estrutura helicoidal única, através de alterações no pH ou força iônica (Gosh *et al.*, 1994). O aumento do pH romperia pontes de hidrogênio entre a molécula de AH e a água, levando à rápida contração da molécula e redução das interações intermoleculares. Esta condição provocaria a perda de estrutura secundária helicoidal e grande relaxamento da cadeia (Gosh *et al.*, 1994). Laurent, (1957) comparou os resultados de viscosidade e espalhamento de luz em soluções de hialuronato de sódio em água e hialuronato de cetilpiridina diluído em metanol. Foi observado um raio de giro de 200 nm para a solução de hialuronato de sódio e de 120 nm para a solução de hialuronato de cetilpiridina, segundo o autor a redução do raio de giro observada para o hialuronato de cetilpiridina está relacionada à quebra das pontes de hidrogênio e relaxação da molécula de AH.

Segundo Reed *et al*., (1989), o pK do AH obtido por extrapolação do grau de ionização é de 2,9, portanto em pH 7,0, esses grupos estão predominantemente ionizados, conferindo caracteristica de poliânion à molécula de AH. Balazs e Laurent, (1951) observaram uma distância aproximada de 1 nm entre as cargas ionizadas dos grupos carboxílicos presentes nos resíduos de ácido D-glicurônico.

Por fim, a característica enovelada e grau de entrelaçamento da molécula de AH é resultado da sua MM, da sua estrutura rígida e das pontes de hidrogênio existentes entre a molécula de AH e a água (Day e Sheehan, 2001).

1.4. PRODUÇÃO MICROBIANA DE AH

A produção do AH por *Streptococcus* é descrita em estudos a mais de 50 anos, *Streptococcus* do grupo de Lancefield do grupo A são considerados patógenos humanos (Kendall *et al*., 1937) e do grupo C (*Streptococcus equi*, *equisimilis* e *zooepidemicus*), patógenos de animais.

Trabalhos iniciais reportados na literatura sobre a produção do AH por via microbiana, utilizaram cepas de bactérias do gênero *Streptococcus*, tais como *S. pyogenes* (Grupo A), *S. equi*, *S. equisimilis*, *S. digalactiae* e *S. zooepidemicus* (Grupo C), conhecidos como hemolíticos por produzirem hemolisina e estreptolisina. Na década de 80 cepas mutantes foram isoladas com a finalidade de produzir AH livre de impurezas tóxicas como estreptolisina e pirogênicos. Foram selecionadas cepas mutantes de *S. zooepidemicus*, NH-131, livre de estreptolisina, depositada no *Fermentation Research Institute* (Akasaka *et al*., 1989), *S. equi* NCIMB 40327 (Ellwood *et al*., 1985) e *S. zooepidemicus* NCTC 7023 (Swann *et al*., 1990).

O microrganismo *Streptococcus equi* subsp. *zooepidemicus* ATCC 39920 utilizado neste trabalho foi desenvolvido na patente de Nimrod *et al*., (1988), onde uma cepa do gênero Streptococcus passaria por um processo mutagênico com nitrosoguanidina, em seguida as espécies mutantes livres de hemolisina foram replicadas em meio sólido composto por agar e sangue de carneiro desfibrinado.

1.4.1 Vias metabólicas do Streptococcus zooepidemicus

A glicose é responsável pela produção das moléculas constituintes da unidade monomérica do AH, a partir da produção de glicose-6-fosfato e frutose-6-fosfato são produzidos ácido D-glicurônico e N-acetilglicosamina respectivamente.

A Figura (1.2) exibe a via biosintética de produção do AH a partir da glicose.

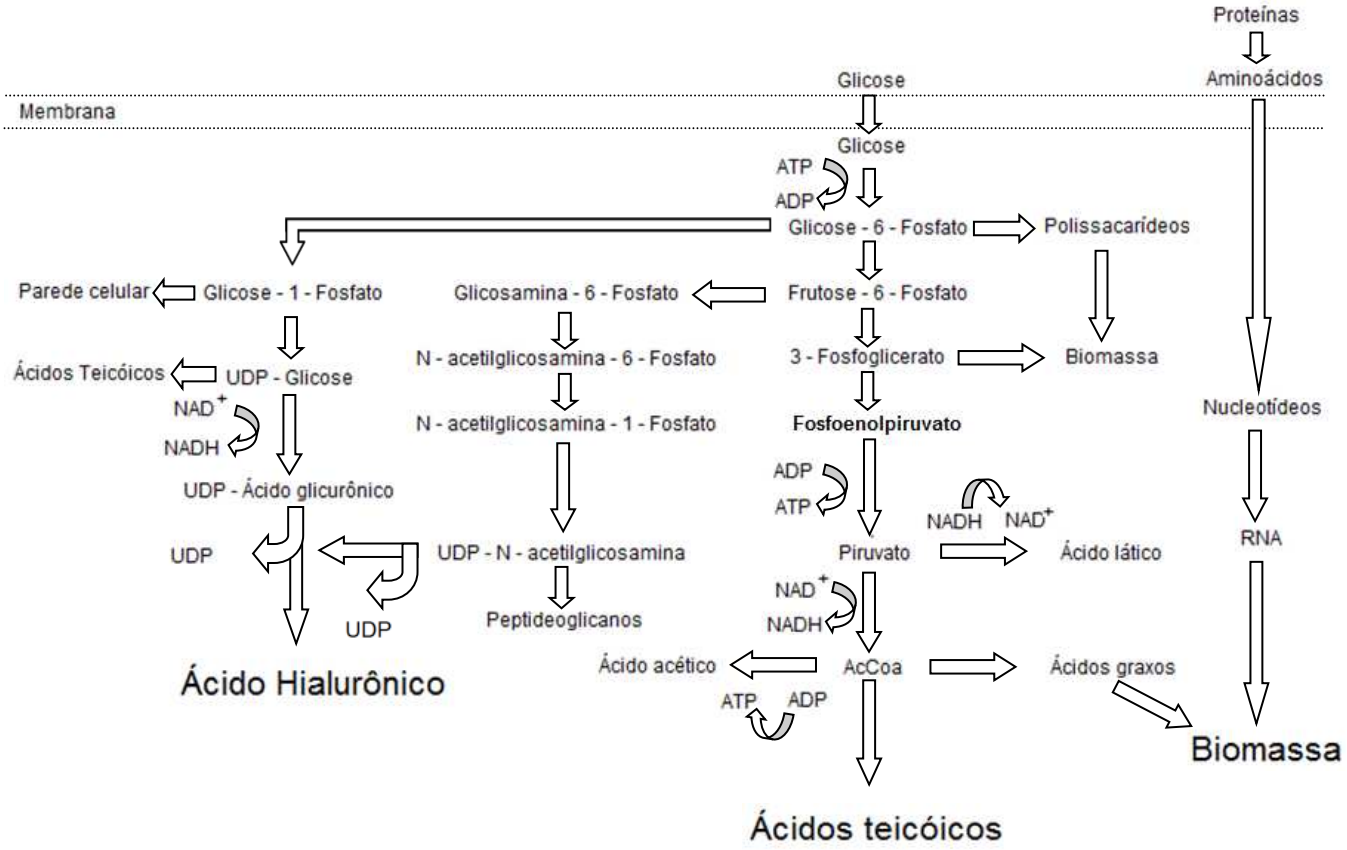

Figura 1.2 - Via biossintética para produção do ácido hialurônico por *Streptococcus zooepidemicus* (adaptado de Liu *et al*., 2011).

Glicose-6-fosfato é convertida em glicose-1-fosfato através da enzima α-fosfoglicomutase, a enzima UDP-glicose pirofosforilase catalisa a reação entre UTP e glicose-1-fosfato produzindo UDP–glicose e através de uma reação de oxidação do grupo álcool primário da UDP-glicose, catalizada pela enzima UDP-glicose desidrogenase, UDP-ácido-glicurônico é produzido (Chong *et al.,* 2005). A partir da frutose–6–fosfato, a enzima amino transferase irá agir na transferência do grupo amina da glutamina para a frutose-6-fosfato produzindo a glicosamina-6-fosfato, em seguida a transferência do grupo acetil através da enzima acetil transferase produz N-acetilglicosamina-6-fosfato (Chong *et al.,* 2005). Um rearranjo do grupo fosfato da molécula N-acetilglicosamina-6-fosfato através de uma mutase produz N-acetilglicosamina-1-fosfato, e através da enzima pirofosforilase, a adição de UDP resulta em UDP–N–acetilglicosamina, por fim, a geração de doadores glicosil ativados viabiliza a polimerização de AH pela enzima hialuronato sintase (Chong *et al.,* 2005).

Glicose-6-fosfato e frutose-6-fosfato fornecem constituintes estruturais da parede celular bacteriana como peptideoglicanos e ácidos teicóicos (Chong *et al*., 2003), portanto a produção de elevados níveis de AH é dependente de níveis adequados de glicose, já que a produção de AH e membrana celular competem pela mesma fonte de carbono (Widner *et.al.,* 2005). Com relação à produção de metabólitos, acetato e lactato são produzidos em maior quantidade através de reações redox do ácido pirúvico (Sedewitz *et al*., 1984). Lactato, formato, acetato e etanol são metabólitos sintetizados a partir das enzimas lactato desidrogenase (LDH), piruvato formato liase (PFL), acetato quinase (AK) e álcool desidrogenase (ADH) respectivamente (Chong e Nielsen, 2003).

O AH é sintetizado através da enzima hialuronato sintase (HAS) para o meio externo na forma de cápsulas (Yamada e Kawasaki, 2005). O mecanismo de biossíntese e elongação da cadeia de AH foi estudado em sistemas enzimáticos livres de células, com mutações no gene da enzima HAS, verificando alterações no comprimento de cadeia do AH (Pummill e DeAngelis, 2003; Rilla *et al*., 2005). A biossíntese do AH é dispendiosa para o microrganismo com relação ao consumo de energia (Chong e Nielsen, 2003). Um total de 4 moles de ATP são consumidos para produzir 1 mol da unidade dissacarídica de AH, sendo 2 moles consumidos em duas reações de glicoquinase para fornecer as hexoses precursoras fosforiladas para cada ramificação da via biosintética, e os outros 2 moles de ATP utilizados para regenerar os doadores UDP (Chong *et al*., 2005). Armstrong e Johns, (1997) observaram que a MM do AH produzido em cultivos possui uma relação negativa com a taxa de crescimento celular e que limitações de glicose causariam primeiramente a queda de produtividade e posteriormente a redução da MM do AH.

1.4.2 Produção de ácido hialurônico por S. zooepidemicus ATCC 39920

A Tabela (1.1) exibe uma série de informações e resultados colhidos na literatura relacionados à produção microbiana do AH em biorreator com aeração forçada.

Tabela 1.1 – Dados de cultivos realizados com aeração forçada utilizando *Streptococcus zooepidemicus* ATCC 39920 para a produção de AH.

Glicose (g/L)	**Fonte de nitrogênio (g/L)**	**Aeração (vvm)** **Agitação (rpm)**	**Fator de Rendimento $Y_{P/X}$ (g/g)**	**Referências**
20	EL - 10	Ae – 1,0 Ag - 200	0,65	(Huang *et al*., 2006)
20	EL - 10	Ag - 300 O.D = 20%	0,75	(Huang *et al*., 2006)
20	EL - 10	Ag - 300 O.D = 20%	0,76	(Chen *et al*., 2008)
40	P – 20 EL - 10	Ae - 0,6 O.D > 30%	0,83	(Duan *et al*., 2008)
20	EL - 10	Ae – 1,0 Ag - 300	0,76	(Wu *et al*., 2009)
40	P - 20 EL - 10	O.D = 10%	1,27	(Duan *et al*., 2009)
10	EL - 10	Ae – 1,0 Ag - 300	0,31	(Don *et al*., 2010)
25	EL - 60	Ae – 2,0 Ag – 250	1,63	(Pires e Santana, 2010)
50	EL - 10	Ae – 1,0 Ag - 300	0,09	(Lai *et al*., 2011)
50	T - 15 EL - 5	Ae – 1,0 Ag - 300	0,22	(Lai *et al*., 2012)
30	EL - 10	Ae – 1,0 Ag - 400	0,80	(Shah *et al*., 2013)

Legenda:
Meio de cultura (Concentrações iniciais): G – Glicose; EL – Extrato de levedura; P – Polipeptona; EC – Extrato de carne; T - Tripeptona
Condições de processo: Ae – Aeração; Ag – Agitação; O.D – Oxigênio dissolvido; Fator de rendimento $Y_{P/X}$: Relação entre as concentrações de AH e biomassa produzidos durante o cultivo

Conforme observado na Tabela (1.1), o fator de rendimento $Y_{P/X}$ varia muito em todos os cultivos, sem uma tendência definida com relação às concentrações das fontes de carbono e nitrogênio, e condições de agitação e aeração utilizadas nos cultivos.

Através de técnicas de recombinação gênica tornou-se possível a transferência do gene responsável pela produção da enzima hialuronato sintase (HAS) em *Streptococcus* para outros microrganismos como *Bacillus subtilis* (Widner *et al*., 2005) e *Escherichia coli* (Yu e Stephanopoulos, 2008) atingindo concentrações de AH próximas a 1,00 g/L e 0,19 g/L respectivamente.

Corsa *et al*., (2016) reportou a produção de 7,5 g/L de AH com massa molar entre 100 e 500 KDa obtida a partir de cultivos com *Bacillus subtilis*. Para o preparo do meio de cultura foram utilizados glicose 4% (m/v), extrato de levedura 2% (m/v) e uma solução composta por arginina 8% (m/v) e histidina 0,4% (m/v). Os cultivos foram realizados em biorreator de 20 L com taxa de agitação e aeração de 1300 rpm e 10-12 L/min respectivamente, controle de pH entre 6,9 to 7,1 em temperatura de 37°C durante 130 horas de cultivo.

1.5. MEIO DE CULTURA

1.5.1 Fontes de carbono e nitrogênio e suas funções

A fonte de carbono utilizada no cultivo do *Streptococcus zooepidemicus* ATCC 39920 normalmente é composta apenas por glicose ou em alguns casos por frutose ou sacarose. A glicose é o substrato responsável pelo fornecimento de energia (ATP) ao microrganismo e pela formação das unidades precursoras do AH (ácido D-glicurônico e N-acetilglicosamina). De acordo com Chong e Nielsen, (2003), a escassez de glicose causaria a redução da produção e MM do AH. Pires *et al*., (2010) estudaram os efeitos metabólicos da concentração inicial de glicose (CIG) em cultivos com *Streptococcus zooepidemicus* ATTC 39920 para a produção do AH. Os ensaios foram realizados com aeração natural (shaker) e forçada (biorreator), e os resultados indicaram que a concentração inicial de glicose a 25 g/L gerou os melhores resultados em termos de produtividade dentre as demais concentrações utilizadas.

Macedo, (2009) observou os efeitos de glicose e frutose extraídos do suco de caju e utilizados como fontes de carbono para a produção do AH através do microrganismo *Streptococcus zooepidemicus* ATCC 39920. Como resultado foi observado uma queda significativa da MM média do AH produzido (10^3 – 10^4 Da), em decorrência da ação do ascorbato presente na fruta.

Pan *et al*., (2017) utilizou melaço de cana de açúcar (85,35 g/L) e extrato de levedura (50 g/L) em cultivos utilizando o microrganismo *Streptococcus zooepidemicus* ATCC 39920 em biorreator de 4,5 L e controle de pH a 8.0. De acordo com os resultados foi obtida uma concentração de 2,82 g/L de AH com MM média de $1,35x10^6$ Da.

Diferentemente da fonte de carbono, a fonte de nitrogênio deve ser composta por uma grande variedade de aminoácidos, em concentrações suficientes para o microrganismo crescer e realizar todas as suas funções (Armstrong *et al*., 1997). Apesar da importância da fonte de carbono para o crescimento celular e formação de produto, é necessário garantir o fornecimento de nitrogênio orgânico, encontrado em quase todas as moléculas orgânicas presentes em organismos vivos. De acordo com a literatura, as fontes de nitrogênio utilizadas na produção de AH são complexas, em sua maioria compostas por extrato de levedura e peptonas de origem animal.

Armstrong *et al*., (1997) verificaram um decréscimo no crescimento do microrganismo *Streptococcus zooepidemicus* ATCC 35246 maior que 50% em relação ao controle, ao variar a concentração de certos aminoácidos considerados essenciais para o desenvolvimento do microrganismo. Ogrodowski *et al*., (2005) demonstraram que o efeito da concentração de extrato de levedura (fonte de nitrogênio) no meio de cultura é mais expressivo que o da glicose (fonte de carbono) para a produção de AH em cultivos com o microrganismo *Streptococcus zooepidemicus* ATCC 39920.

Além da preocupação com a quantidade e qualidade do AH produzido, existe também uma grande preocupação com a qualidade do meio de cultura utilizado. Nos últimos 10 anos as indústrias farmacêuticas e de cosméticos têm optado pelo emprego de meios de cultura de origem vegetal por motivos de segurança. No caso do extrato de levedura, o elevado teor de ácidos nucléicos e RNA pode oferecer restrições ao seu uso em se tratando do AH aplicado na área médica (Von Der Haar, 2007).

Alguns estudos reportam os benefícios da utilização da peptona de soja como fonte de nitrogênio em processos fermentativos (Heenan *et al*., 2002; Liu *et al*., 2005; Patel *et al*., 2008).

De acordo com a definição de Green *et al.,* (1977), peptonas são hidrolisados de proteínas solúveis em água. As peptonas comerciais utilizadas em meios microbiológicos são principalmente derivadas da caseína, soja e carne. Benedini e Santana, (2012) comprovaram a eficiência da utilização da peptona de soja como única fonte de nitrogênio, desde a preparação do inóculo em placas de Petri até a fermentação propriamente dita. A utilização da peptona de soja no preparo do inóculo em placas de *Petri* resultou em um valor de rendimento ($Y_{P/X}$) próximo ao do meio contendo BHI (*Brain and Heart Infusion*) e sangue de carneiro desfibrinado utilizado como controle. Além da substituição de fontes de nitrogênio de origem animal como o BHI e o sangue de carneiro, a peptona de soja trouxe vantagens como a redução do teor de PPT (proteínas e peptídeos totais) e a remoção de compostos de origem animal, reduzindo os riscos de reações imunogênicas e efeitos colaterais provocados pelo AH impuro.

1.5.2 RAZÃO C/N

A razão carbono/nitrogênio (C/N) é muito importante na biossíntese do AH. Estirpes de *Streptococcus zooepidemicus* são dependentes de carbono e de nitrogênio para o crescimento e produção de AH (Armstrong *et al*., 1997). Chen *et al*., (2009) encontraram uma razão C/N ideal de 2,0 para o cultivo de *Streptococcus zooepidemicus* ATCC 39920 em ensaios com aeração forçada e controle de pH. O meio de cultura utilizado era composto por glicose e extrato de levedura. Lai *et al*., (2012) encontraram uma razão C/N ideal de 2,5, utilizando o mesmo microrganismo, em meio de cultura composto por glicose, extrato de levedura e triptona, em cultivos com aeração forçada e controle de pH.

No entanto nesses dois últimos estudos, as razões C/N calculadas eram referentes às concentrações das fontes de carbono e nitrogênio em vez da concentração de carbono presente na fonte de carbono e da concentração de nitrogênio e carbono presentes na fonte de nitrogênio complexa. Além da razão C/N, a presença de aminoácidos específicos é muito importante, e isto se deve ao fato de que certos aminoácidos em excesso podem dificultar a absorção de um aminoácido considerado essencial para a produção de AH (Guirard e Snell, 1962). A glutamina por exemplo é utilizada na síntese de N-acetilglicosamina, um dos precursores do AH, e também pode ser encontrada na parede celular do microrganismo produtor (Chong *et al*., 2005).

Oliveira, (2014) observou alterações na produção e principalmente na MM do AH, decorrentes da utilização de dois tipos de peptona de soja compostas por diferentes proporções de aminoácidos livres (AL). A importância relacionada à essas proporções se

mostrou evidente a partir do momento que uma mesma razão C/N (4,1) apresentou diferentes resultados de rendimento e MM do AH obtido. O AH produzido com a peptona de soja rica em aspartato, glutamina e glutamato (AGG) apresentou MM média de 1,16 x 10^6 Da, enquanto que o AH produzido com a peptona pobre em AGG apresentou MM média de 8,9 x 10^3 Da. Sendo assim a especificação da razão C/N e do perfil de AL utilizados no meio de cultura é de fundamental importância para o entendimento e reprodução dos resultados que envolvam a produção do AH por via microbiana.

1.5.3 CARDIOLIPINA

A cardiolipina é um fosfolípido com estrutura dimérica contendo quatro grupos acil, encontrada exclusivamente em membranas bacterianas e mitocondriais, com a função de gerar um potencial eletroquímico para o transporte de substratos e síntese de ATP (Shlame *et al*., 2000). O nome "cardiolipina" faz referência ao tecido do qual foi isolado pela primeira vez em 1942 (Pangborn, 1942), e preparações comerciais de cardiolipina são derivadas do tecido cardíaco até os dias de hoje. A função da cardiolipina na membrana celular está relacionada com a sua capacidade única de interação com proteínas (Shlame *et al*., 2000).

Como a membrana celular bacteriana é constituída de uma parte hidrofóbica e uma parte hidrofílica, Weigel, (1998) propôs que a cardiolipina agiria juntamente com a enzima hialuronato sintase (HAS) de *Stretptococcus* na extrusão do AH, ou seja, a parte lipídica da cardiolipina interagiria com a bicamada lipídica da membrana celular bacteriana e as porções hidrofóbicas da cadeia de AH, enquanto os grupos acídicos da cardiolipina interagiriam com a enzima e as porções hidrofílicas da cadeia do AH, desta forma, os fosfolipídios e as enzimas criariam uma passagem na membrana na forma de poro, através do qual o AH sintetizado seria mais facilmente extrudado.

Segundo Tlapak-Simmons *et al*., (1998) a enzima HAS necessita aproximadamente de 16 moléculas de cardiolipina para se tornar ativa, verificou-se também que esta enzima apresenta baixa atividade quando purificada sem a presença da cardiolipina, na presença da cardiolipina a atividade da enzima HAS é elevada em 30%. A cardiolipina em contato com a membrana celular se intercala rapidamente, sendo capaz de interagir e ativar as enzimas que estão parcialmente ou completamente inativas (Tlapak-Simmons *et al*., 1998).

1.6. CONDIÇÕES OPERACIONAIS

1.6.1 POTENCIAL HIDROGENIÔNICO (PH)

Existem poucos estudos na literatura relacionados à influência do pH na produção do AH, Stoolmiller e Dorfman, (1969) relatam a máxima atividade da enzima HAS em pH próximo a 7,0, através de estudos utilizando a membrana de *Streptococcus equisimilis.* Armstrong e Johns, (1997) observaram aumento considerável na produção do AH quando o pH do meio de cultivo foi controlado a 7,0, no entanto nenhuma alteração significativa foi observada na MM do AH produzido.

Liu *et al*., (2009) utilizou uma estratégia de estresse alcalino intermitente a pH 8,5 com o microrganismo *Streptococcus zooepidemicus*. O valor de pH foi mantido a 7,0 durante as primeiras 6 horas, após isso o pH alternou entre 8,0 (durante 1 hora) e 7.0 (durante 1 hora) até o fim do cultivo com 16 horas de duração. Com a estratégia intermitente de estresse alcalino, a produção de AH aumentou 30% em relação ao controle, no qual o pH foi mantido em 7,0 durante todo o cultivo. Pan *et al*., (2017) utilizou controle de pH a 8,0 em cultivos com o microrganismo *Streptococcus zooepidemicus* ATCC 39920, em biorreator de 4,5 L e meio de cultura composto por melaço de cana de açúcar (85,35 g/L) e extrato de levedura (50 g/L). Foi obtida uma concentração de 2,82 g/L de AH após 24 horas de cultivo, valor 2,86 vezes maior que o obtido pelo cultivo sem controle de pH.

1.6.2 AGITAÇÃO E AERAÇÃO

Bactérias do gênero estreptococos são homoláticas e anaeróbias facultativas, porém são incapazes de realizar fosforilação oxidativa, sendo assim metabolizam anaerobicamente a glicose através da via glicolítica Embden-Meyerhof. Sob condições anaeróbicas produzem lactato como produto principal a partir do catabolismo da glicose, juntamente com pequenas concentrações de acetato, etanol e formato.

Condições aeróbias poderiam ser benéficas para a produção de AH. Este fenômeno pode ter diferentes explicações: (1) O oxigênio pode estimular a síntese de AH como forma de proteção contra os efeitos nocivos do oxigênio (Cleary e Larkin, 1979; Chong e Nielsen, 2003); (2) O oxigênio dissolvido (O.D) no meio pode redirecionar parte do fluxo de carbono para a produção de acetato em vez de ácido láctico com produção extra de ATP ($Y_{ATP/glicose}$ é de 3 mol/mol com a produção de acetato, contra 2 mol/mol com a produção de lactato) com maior energia disponível para a produção de AH (Gao *et al*., 2006; Chong e Nielsen, 2003).

(3) A aeração poderia estimular a produção de acetil-CoA e este componente em excesso poderia ser desviado para reabastecer o acetil-CoA utilizado na síntese do AH (Wu *et al*., 2009).

Chong e Nielsen, (2003) utilizaram a super-expressão de NAD^+ para elevar a produção de ATP através da produção de ácido acético, utilizando o microrganismo *Streptococcus zooepidemicus* ATCC 35246 em cultivos realizados em shaker e meio de cultura contendo glicose (5,0 g/L) e glutamina (5,6 g/L). Como resultado houve crescimento da biomassa e pouca alteração na produção de AH. Gao *et al*., (2006) chegaram a mesma conclusão quando diferentes taxas de O.D foram introduzidas no meio de cultura contendo glicose (20 g/L) e extrato de levedura (20 g/L), em cultivos com o microrganismo *Streptococcus zooepidemicus* H23, em biorreator de 2,5 L.

Foi observado que a taxa de crescimento celular é inversamente proporcional a MM do AH (Armstrong e Johns, 1997; Chong *et al*., 2005). O aumento da taxa específica de crescimento traz o aumento da atividade da proteína sintetizante (PSS), que seria responsável pelo aumento da concentração da enzima hialuronato sintase na membrana celular, e a alta densidade dessa enzima não seria benéfica para o aumento da MM do AH (Sheng *et al*., 2009).

O estabelecimento de diferentes taxas de O.D visando à otimização da MM e produção do AH foi tema de diversos trabalhos. Duan *et al.*, (2009) concluiu que O.D acima de 10% da saturação não resultaria em variações significativas na produção de AH, porém, até 50% (O.D), houve o aumento da MM do AH e a partir deste valor haveria degradação da cadeia devido à presença de espécies reativas de oxigênio. A redução da MM do AH a partir de espécies reativas de oxigênio foi atribuída ao aumento dos níveis de O.D, proporcionado pelo controle da agitação segundo muitos autores (Praest *et al*., 1997 ; Jagannath, e Ramachandran, 2010 ; Gao *et al*., 2003). A ação de radicais livres poderia prejudicar a MM do AH, porém a adição de antioxidantes reverteria esse processo. Os ácidos tânico, oxálico e salicílico poderiam ser utilizados para reverter o processo causado por radicais livres segundo Shah *et al*., (2013).

Kim *et al*., (1996) observaram que o aumento da agitação (400 – 1200 rpm) provocou redução da biomassa e da produção de AH, porem a MM aumentou consideravelmente. Armstrong e Johns, (1997) observaram que a MM do AH era independente da agitação, sugerindo que a molécula de AH seria resistente às forças cisalhantes produzidas pelo impelidor. Liu *et al*., (2009) utilizaram três estágios de agitação em etapas diferentes do cultivo, 200 rpm (0 – 8h), 400 rpm (8 – 12h), 600 rpm (12 – 20h) a 0,5 vvm, e verificaram um

aumento de 10% na concentração do AH em relação ao controle com taxa de aeração de 0,5 vvm e agitação de 200 rpm. Huang *et al*., (2006) verificaram que sob agitação homogênea, o aumento da taxa de O.D (acima de 5%) não apresentou efeito algum sobre o crescimento celular e produção do AH. Jagannath e Ramachandran, (2010) utilizaram o microrganismo *Streptococcus zooepidemicus* ATCC 39920 em biorreator de 3,7 L, meio de cultura contendo meio quimicamente definido composto glicose (30 g/L), sais e aminoácidos diversos para a biossíntese do AH. A biomassa e o rendimento do AH aumentaram em cerca de 25% e 13% respectivamente, quando a taxa de O.D foi mantida a 30% da saturação através da aeração constante de 1 vvm e do controle da agitação (intervalo de agitação: 200-800 rpm), sem alterações significativas na MM do AH.

1.7. PURIFICAÇÃO

A combinação de diferentes operações para a purificação do AH é fundamental para o aumento da eficiência e rendimento na remoção de impurezas. As principais operações utilizadas na purificação do AH são: precipitação (Cleland e Sherblom, 1977); filtração (diafiltração e ultrafiltração) (Zhou *et al.*, 2006; Oueslati *et al.*, 2015) e adsorção (Rajendran *et al*., 2016).

A precipitação do AH com solvente orgânico é uma das operações mais utilizadas para a remoção de proteínas do caldo fermentado. Etanol, metanol, acetona e propanol promovem a redução da constante dielétrica do meio provocando o aumento das interações eletrostáticas entre moléculas de proteínas e AH (Scopes, 1988).

A filtração tangencial é uma operação bastante utilizada na remoção de impurezas do AH produzido por via microbiana. Zhou *et al*., (2006) utilizaram a microfiltração e a ultrafiltração tangencial na remoção de biomassa e proteínas do caldo fermentado respectivamente. Foram utilizadas membranas de fluoreto de polivinilideno com diâmetro de poro entre 0,20 e 0,45 μm na etapa de microfiltração e membranas com diâmetro de poro entre 100 kDa e 300 kDa na etapa de ultrafiltração. A solução purificada de AH apresentou concentração de proteínas em torno de 0,07% m/v, com recuperação de 77% da concentração inicial de AH.

A adsorção é um fenômeno físico-químico onde um determinado componente da fase líquida fica retido na superfície de uma fase sólida. O componente aderido à superfície é chamado de adsorvato, enquanto que a fase sólida que retém o adsorvato é chamada adsorvente. A área superficial do adsorvente é um parâmetro muito importante, pois quanto

maior esta área maior será a eficiência da adsorção. Por isso, partículas adsorventes com elevada porosidade são muito utilizadas em etapas de purificação.

Para a purificação de AH a adsorção é normalmente utilizada após as etapas de precipitação e filtração. O carvão ativado é o adsorvente mais utilizado devido ao baixo custo e elevada eficiência. O carvão ativado é um material poroso com formato microcristalino, possui grande porosidade e área superficial. A maioria dos materiais carbonáceos possui área superficial entre 10 e 15 m^2/g, após a oxidação dos átomos de carbono o carvão ativado passaria a ter área superficial acima de 800 m^2/g (Boudou, 2003).

Rajendran *et al*., (2016) utilizaram *Lactococcus lactis* NZ9020 para produzir o AH em meio de cultura composto por 5 g/L de BHI e 5 g/L de extrato de levedura. O caldo fermentado foi tratado com 1% (m/v) de carvão ativado durante 2 horas de agitação contínua, removendo proteínas remanescentes e impurezas. A concentração final de proteínas e ácidos nucleicos na solução obtida após a adsorção foi quase insignificante, com perdas de AH entre 8 e 10% m/v. Choi *et al*., (2014) utilizaram *Streptococcus zooepidemicus* em cultivos com extrato de levedura (15 ~ 25 g/L) e glicose (60 ~ 80 g/L) em biorreator de 5 L com taxa de agitação de 300 rpm durante 25 horas. Carvão ativado a 2,00% (m/v) foi utilizado para remoção de cerca de 96% das proteínas e peptídeos totais e 90% das endotoxinas provenientes do caldo fermentado.

MATERIAIS E MÉTODOS

2.1. MICRORGANISMO

Foi utilizada neste trabalho a cepa de *Streptococcus equi* subsp. *zooepidemicus* ATCC 39920, pertencente ao grupo C de Lancefield, selecionada previamente por Ogrodowski,(2002) para a produção do AH. A cepa nativa foi obtida através da *American Type Culture Collection* (ATCC) na forma de cultura liofilizada e depositada na Coleção Brasileira de Microrganismos de Ambiente e Indústria (CBMAI).

2.2. MANUTENÇÃO DA CULTURA

A partir da cepa obtida na ATCC o conteúdo liofilizado foi hidratado em água deionizada e estéril, seguido de plaqueamento em *TSA* (*Trypticase Soy Agar*) e incubação por 24 horas a 37ºC. Para a preparação do estoque de culturas ultracongeladas, as colônias foram transferidas para criotubos contendo 0,5 mL de uma solução de glicerol 10%, completando 1 mL da suspensão. Os criotubos foram armazenados a -20ºC por 20 minutos, em seguida os criotubos foram transferidos para o ultrafreezer a -80ºC onde foram preservados. Os criotubos obtidos neste procedimento foram denominados a primeira geração da cultura.

Um segundo estoque de criotubos foi preparado a partir da primeira geração. Para tanto, um criotubo da primeira geração foi descongelado e a suspensão de células estriada em placas com ágar e peptona de soja (67 g/L) (Benedini e Santana, 2012). As placas foram incubadas por 24h a 37ºC e os procedimentos subseqüentes para preparação dos criotubos foram os mesmos descritos anteriormente para a primeira geração. O segundo estoque de criotubos foi denominado segunda geração da cultura.

Neste trabalho foram empregadas culturas propagadas a partir da segunda geração.

2.3. MEIO DE CULTURA

Foi utilizada como fonte de carbono a glicose e como fonte de nitrogênio as peptonas de soja; Soy Peptone E-110, (Organotechnie, La Courneuve, France), Hy–soy, (Kerry Inc Millington Road Beloit, WI, USA) e Hy-Soy® T - P6463 (Sigma-Aldrich Co, Saint Louis, MO, USA). Extrato de levedura (Oxoid LTD, Hampshire, UK) foi utilizado como controle.

2.3.1 *CARBONO ORGÂNICO TOTAL*

A concentração de carbono orgânico total (COT) presente na peptona de soja foi determinada utilizando o equipamento TOC-L (Shimadzu, Quioto, Japão), através do método de combustão oxidativa catalítica (Sugimura e Suzuki, 1988). Este método realiza a combustão total das amostras, aquecendo-as a 680°C em um ambiente rico em oxigênio, envolvendo tubos de combustão preenchidos com catalisador de platina. O dióxido de carbono gerado pela oxidação é detectado através de um analisador de gás infravermelho (NDIR).

2.3.2 *NITROGÊNIO TOTAL*

Determinou-se o teor de proteínas e peptídeos totais (PPT) através do método BCA (Ácido Bicinconínico) (Pierce Chemical Company / Thermo Scientific, Rockford, IL). Nitrogênio *Kjeldahl* foi calculado dividindo-se o teor de PPT pelo fator 6,25, de acordo com a portaria nº. 360 da Anvisa.

2.4. INÓCULO

Uma ampola contendo a cultura de *Streptococcus equi subsp, zooepidemicus* ATCC 39920 foi descongelada e estriada em placas de *Petri* contendo peptona de soja (67 g/L) e ágar bacteriológico (Benedini e Santana, 2013). As placas foram incubadas a 37°C durante 24 horas; em seguida as colônias são transferidas para frascos Erlenmeyer contendo meio de cultura composto por glicose e peptona de soja ou glicose e extrato de levedura. Para os cultivos realizados em shaker, os frascos foram incubados sob agitação de 150 rpm, a 37ºC, durante 12 horas. Para os cultivos em biorreator uma segunda etapa de inóculo é necessária, com duração de 6 horas e um aumento de 10 vezes em volume de meio de cultura entre o primeiro e segundo inóculo.

2.5. FERMENTAÇÃO

O inóculo foi transferido a 10% (v/v) para frascos Erlenmeyer ou biorreator. Temperatura é mantida a 37°C durante 24 ou 20 horas de cultivo, controle de pH a 7,0 foi realizado com uma solução de NaOH (20% m/v). Concentração de biomassa, AH, glicose e metabólitos foram determinados em amostras retiradas de duas em duas horas nos cultivos em biorreator e nos pontos iniciais e finais dos cultivos em shaker. As análises dessas amostras foram realizadas em triplicata.

A Tabela (2.1) exibe informações relacionadas ao meio de cultura e controle dos cultivos utilizados nos artigos 1, 2, 3 e 4.

Tabela 2.1 - Dados dos cultivos realizados com *Streptococcus zooepidemicus* ATCC 39920 e utilizados nos artigos 1, 2, 3 e 4.

	FC	FN	CL (mg/L)	Razão G/N (g/g)	AA (L/min)	OP (L/min)	TA (rpm)	O.D (%)	pH
Artigo 1	G (25 g/L)	PS[a]	-	4,3	5,0	-	250	-	-
	G (25 g/L)	PS[b]	-	4,3	5,0	-	250	-	-
	G (25 g/L)	PS[c]	-	4,3	5,0	-	250	-	-
Artigo 2	G (25 g/L)	EL	0,0	4,3	-	-	150	-	-
	G (25 g/L)	EL	10,0	4,3	-	-	150	-	-
	G (25 g/L)	EL	20,0	4,3	-	-	150	-	-
	G (25 g/L)	EL	30,0	4,3	-	-	150	-	-
	G (25 g/L)	PS[b]	0,0	4,3	-	-	150	-	-
	G (25 g/L)	PS[b]	30,0	4,3	-	-	150	-	-
Artigo 3	G (25 g/L)	PS[b]	-	4,3	0,0	-	250	-	7,0
	G (25 g/L)	PS[b]	-	4,3	5,0	-	250	-	7,0
	G (25 g/L)	PS[b]	-	4,3	5,0	0,5	250	-	7,0
	G (25 g/L)	PS[b]	-	4,3	-	-	-	90	7,0
	G (70 g/L)	PS[b]	-	12,0	-	-	-	90	7,0
	G (70 g/L)	PS[b]	-	12,0	-	-	-	90 (Após 3 horas)	7,0
Artigo 4	G (25 g/L)	PS[b]	-	4,3	-	-	-	90	7,0

FC – Fonte de carbono (G – Glicose); FN – Fonte de nitrogênio (EL – Extrato de levedura; PS – Peptona de soja; a - E-110 – Organotechnie; b - Hy-Soy – Kerry; c - Hy-Soy T - Sigma)
CL – Cardiolipina; G/N – Glicose/Nitrogênio; AA – Ar atmosférico; OP – Oxigênio puro; TA – Taxa de agitação; O.D – Oxigênio dissolvido

ARTIGO 1 – "The amino acids profile soy-peptone as a determinant of the molar mass of hyaluronic acid synthesized by *Streptococcus zooepidemicus*" ARTIGO 2 - "Cardiolipin induces the production of high molar mass hyaluronic acid from *Streptococcus zooepidemicus* cultivation", ARTIGO 3 - "The oxygen control modulates high microbial production and molar mass of hyaluronic acid", ARTIGO 4 - "Effect of pH on adsorption with activated carbon for purification of hyaluronic acid"

A Figura (2.1) exibe o BIOFLO III de 3 L (New Brunswick Scientific Co, Inc, Edison, NJ, EUA) utilizado nos cultivos com vazão fixa de oxigênio e taxa de agitação.

Figura 2.1 – Biorreator BIOFLO III de 3 L (New Brunswick Scientific Co, Inc, Edison, NJ, EUA) utilizado nos cultivos com valores fixos de vazão de oxigênio e taxa de agitação.

Para os cultivos com controle de O.D, um controlador híbrido (PID + Heurística) foi utilizado através do controle automático da taxa de agitação e composição da corrente de gás fornecida ao biorreator (5 L) através dos fluxômetros (GFC ALLBORG) de oxigênio e ar atmosférico. Um eletrodo de oxigênio (Mettler Toledo Inpro 6800) conectado a um transmissor (CE O_2 4050) foi utilizado. As concentrações de gás carbônico e oxigênio foram quantificadas na saída do biorreator através de um analisador Sick/Maihak S.710. A aquisição dos dados de cultivo foi realizada em um intervalo de tempo de 10 segundos através do software SuperSys_HCDC^R (Horta et al., 2014). A Figura (2.2) exibe a relação de equipamentos utilizados nos cultivos com controle de O.D.

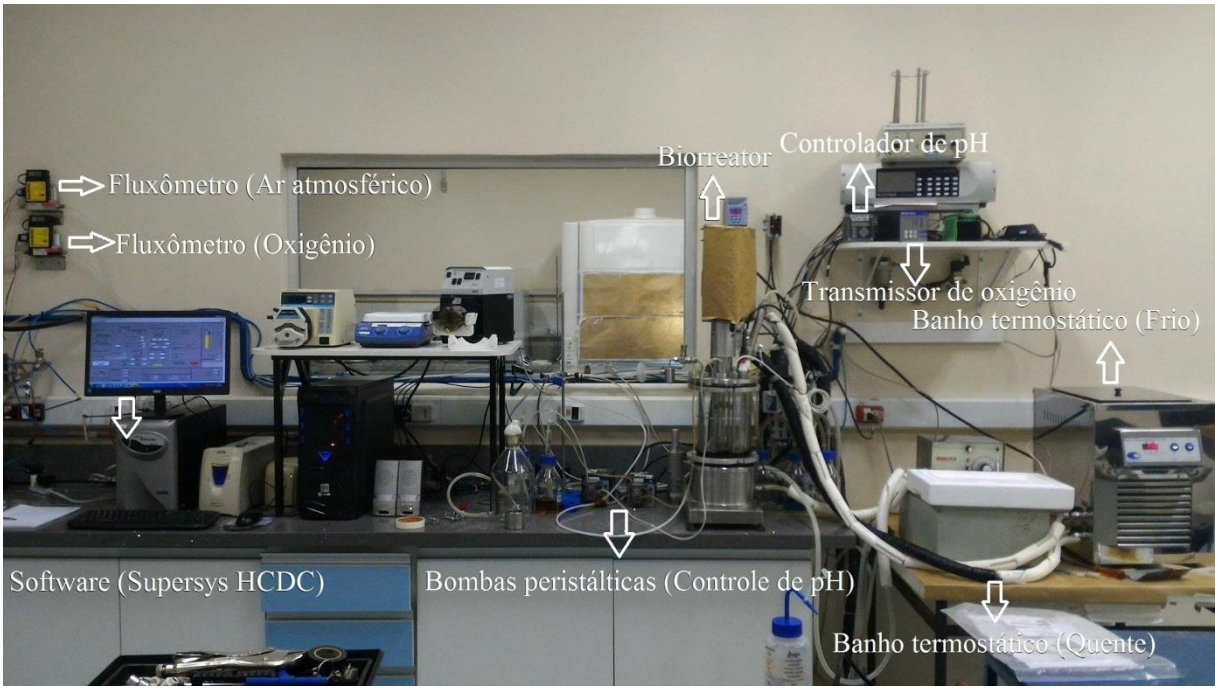

Figura 2.2 – Equipamentos utilizados nos cultivos com controle de oxigênio dissolvido.

2.6. RECUPERAÇÃO E CONCENTRAÇÃO DO AH

O caldo fermentado foi centrifugado em tubos *Falcon* a 3200 rpm durante 20 min para a separação da biomassa. Etanol é adicionado ao sobrenadante a uma razão de 1,5/1 v/v (etanol/sobrenadante). Em seguida a solução é mantida a 4°C durante 1 hora para a precipitação do AH seguido de centrifugação a 3200 rpm durante 20 min. Uma solução de NaCl (0,15 mol/L) é adicionada ao precipitado e a solução é ressuspendida. Os processos de adição de etanol, refrigeração, centrifugação e diluição em NaCl 0,15 M foram realizados mais duas vezes.

2.7. MÉTODOS ANALÍTICOS

2.7.1 CRESCIMENTO CELULAR

Para a quantificação da biomassa foi utilizado o método gravimétrico, portanto o caldo fermentado foi centrifugado em tubos *Falcon* previamente pesados. Após a centrifugação, o sobrenadante é separado e o precipitado composto por biomassa é lavado, ressuspendido com água destilada e centrifugado mais duas vezes. Por fim, os tubos são secos e pesados.

2.7.2 *CONCENTRAÇÃO DE AH*

O método turbidimétrico foi aplicado para determinar a concentração de AH nas amostras (Chen e Wang, 2009). Para a construção da curva de calibração, AH padrão (Genzyme Corporation, Cambridge, MA, EUA) foi utilizado com leituras de absorbância a 400 nm.

2.7.3 *CONCENTRAÇÃO DE GLICOSE*

O kit comercial glicose oxidase (Laborlab Ltda, Guarulhos, São Paulo, Brasil) foi utilizado para determinar a concentração de glicose no caldo fermentado. Teste enzimático e colorimétrico composto pela enzima Glicose Oxidase-Peroxidase com leituras de absorbância a 505 nm.

2.7.4 *CONCENTRAÇÃO DE METABÓLITOS*

Para a quantificação de acetato e lactato, as amostras foram inicialmente filtradas em membranas com diâmetro de poro de 0,2 um (Sartorius, Goettingen, Alemanha). As amostras filtradas foram injetadas no HPLC (Shimadzu Corporation, Kyoto, Japão) ligado a uma coluna de troca iônica HPX-87H (Aminex, Bio-Rad, 70 Hercules, CA, EUA) com dimensão de 7,8 mm x 300mm. A fase móvel é composta de H_2SO_4 (0,004 M) com vazão constante de 0,6 mL/min (Chong e Nielsen, 2003). O sinal é monitorado por um detector de índice de refração (RID-6A, Shimadzu Corporation, Quioto, Japão) e a temperatura mantida a 65°C.

2.7.5 *MASSA MOLAR*

A MM média ponderal do AH produzido foi determinada por um sistema cromatográfico de exclusão de tamanho (Shimadzu Corporation, Kyoto, Japão) conectado a uma pré coluna (PolySep-GFC-P, 7,8 mm x 35mm, Phenomenex, Torrance, CA, EUA) e uma coluna de filtração em gel (PolySep-GFC-P6000, 7,8 mm x 300 mm; Phenomenex, Torrance, CA, EUA). O perfil dos picos foi monitorado através de um detector de índice de refração (RID-6A, Shimadzu Corporation, Quioto, Japão). A Eq. 2.1 exibe a equação obtida a partir da curva de calibração construída com padrões de AH com MM de 50, 150, 250, 500 e 1000 KDa (R^2 – 0,9822).

$$Log\ (MM) = 10{,}947 - 0{,}619\ (Tempo\ de\ retenção) \qquad 2.1$$

O cálculo de distribuição da massa molar foi realizado através da divisão por área dos picos cromatográficos com auxílio do software LCsolution 1.0.0.1 (Shimadzu Corporation, Quioto, Japão).

Resultados e Discussão

Este capítulo será apresentado na forma de artigos submetidos à períodicos científicos, além do resumo de uma patente (BR 10 2017 027243 50) envolvendo a produção e purificação do ácido hialurônico.

Artigo 1	The amino acids profile soy-peptone as a determinant of the molar mass of hyaluronic acid synthesized by *Streptococcus zooepidemicus*
Artigo 2	Cardiolipin induces the production of high molar mass hyaluronic acid from *Streptococcus zooepidemicus* cultivation
Artigo 3	Oxygen control modulates microbial production and molar mass of hyaluronic acid synthesized by *Streptococcus zooepidemicus*
Artigo 4	Effect of pH on adsorption with activated carbon for purification of hyaluronic acid
Patente	Processo para produção e purificação de ácido hialurônico por via microbiana

Artigo 1

THE AMINO ACIDS PROFILE SOY-PEPTONE AS A DETERMINANT OF THE MOLAR MASS OF HYALURONIC ACID SYNTHESIZED BY *STREPTOCOCCUS ZOOEPIDEMICUS*

ARTIGO SUBMETIDO AO PERIÓDICO

BIORESOURCE TECHNOLOGY

The amino acid profile of soy-peptone as a determinant of the molar mass of hyaluronic acid synthesized by *Streptococcus zooepidemicus*

Rhelvis de C. Oliveira[1] and Maria H. A. Santana[1*]

[1]Department of Engineering of Materials and Bioprocesses, School of Chemical Engineering, University of Campinas, Albert Einstein Avenue, 500, Campinas - SP, 13083-852.

Abstract Soy peptones (SPs) are promising plant resources for hyaluronic acid (HA) production due to their reduced immunogenic effects compared with those of yeast extract, which is currently used in HA biosynthesis. The present study examined the influence of the amino acid profile of three commercial SPs on HA molar mass (HA-MM). Batch cultivations with forced aeration were performed with *Streptococcus zooepidemicus* ATCC 39920. The free to total amino acid ratio ($FATA^{-1}$) as well as the presence of glutamine and glutamate determined the HA-MM due to the balance of the HA precursor monomers. A higher FA fraction reduced the specific growth rate, thus channeling the released energy into increasing the HA-MM. Therefore, the SP amino acid profile plays an important role in HA-MM control and can be used to meet requirements for specific medical applications.

Keywords Hyaluronic acid - *Streptococcus zooepidemicus* - Vegetable peptones - Submerged fermentation - Nitrogen sources

M. H. A. Santana*

1 - University of Campinas, School of Chemical Engineering, Department of Materials Engineering and Bioprocess, 500, Albert Einstein Avenue, 13083-852 - Campinas - SP - Brazil

Email: mariahelena.santana@gmail.com, Tel.: +55 19 3521 3921, fax: +55 19 3521 3890

1. INTRODUCTION

Hyaluronic acid (HA) is a natural polysaccharide with a wide range of biological functions that is composed of repetitive disaccharide units of D-glucuronic acid (GlcUA) and N-acetyl-glucosamine (GlcNAc), interconnected through β-1,3- and β-1,4-glycosidic bonds between the monomer and disaccharide units, respectively. HA is present in high concentrations in cartilage, vitreous humor, synovial fluid and human skin. Approximately 35% of the HA present in humans is found in the muscles and skeleton (Chong et al., 2005).

Intrinsically, HA provides viscoelastic properties to synovial fluid in joints and skin. Exogenous HA has been used as a therapeutic tool to restore the decline of endogenous HA caused by aging and diseases. The best effects are achieved when the HA molar mass (HA-MM) is high, and the safest HA is derived from biological cultures. Therefore, the quality of HA for medical applications is determined by its purity and HA-MM, which can range between 10^4 and 10^7 Da (Armstrong and Johns, 1997).

Commercial HA are derived from an avian source (rooster combs), or they are produced microbially. Bacteria of the genus *Streptococcus* synthesize HA as a protective mechanism (DeAngelis, 1999), producing a thick layer of it around cells. Therefore, changes in the microbial environment can increase HA production (Duan et al., 2009).

Previous studies have shown that HA biosynthesis is associated with cell growth, but both processes compete for carbon sources and energy during cultivation (Ellwood et al., 1996). Chong et al. (2005) reported that HA biosynthesis requires substantial energy consumption: 4 mol of ATP per 1 mol of HA disaccharide repeating unit (GlcUA and GlcNAc) produced. Therefore, an adequate balance between the carbon (glucose) and nitrogen sources (yeast extract) could lead to increased HA yields in relation to biomass (Ogrodowski et al., 2005; Zhang et al., 2006).

Armstrong and Jonhns (1997) described the effect of the initial glucose concentration on the HA-MM produced by *Streptococcus zooepidemicus* ATCC 35246 in a 2-L bioreactor under anaerobic conditions, using a culture medium composed of glucose and 10 g L^{-1} yeast extract. By increasing the initial glucose concentration from 20 to 40 g L^{-1}, the average HA-MM increased approximately 33%. How the initial glucose concentration affects the cultivation of *Streptococcus zooepidemicus* ATCC 39920 was also studied by Pires et al. (2010a) in Erlenmeyer flasks (natural aeration) and 3.0L bioreactor (forced aeration). The results indicated that an initial glucose concentration of 25 g L^{-1} and a glucose/nitrogen (G/N) ratio of 4.3 were the best conditions for HA production with yeast extract as the nitrogen source.

Jagannath and Ramachandran (2010) reported the effects of changes in the carbon and nitrogen source concentrations when cultivating *Streptococcus zooepidemicus* ATCC 39920 in 3.7 L bioreactor with a culture medium composed of glucose (30 g L^{-1}) and yeast extract (10 g L^{-1}). Adding N-acetylglucosamine (2 g L^{-1}) and pyruvate (10 g L^{-1}) to the culture medium enhanced the HA-MM by 81% and 10% and improved HA production by 45% and 46%, respectively. With respect to the nitrogen source, the addition of glutamine (5 g L^{-1}) significantly increased the specific growth rate (81%) as well as HA production (11%) without affecting the HA-MM.

According to Chong et al. (2005) glutamine is used in the synthesis of N-acetyl-glucosamine, which is one of the precursors of HA. The amino group of glutamine is transferred to fructose-6-phosphate via an amido transferase, producing glucosamine-6-phosphate. N-acetylglucosamine-6-phosphate is produced through the addition of an acetyl group by an acetyltransferase. After phosphate group rearrangement, N-acetyl glucosamine-1-phosphate is produced, leading to the intermediate UDP-N-acetylglucosamine.

Free (FAs) and total (TAs) amino acids in complex nitrogen sources can directly affect their transport efficiency into cells, because di- and tripeptides in the TA fraction can affect cell growth more than a corresponding amount of FAs (Guirard and Snell, 1962). According to Law et al. (1976), data from a streptococci growth study indicated that the total uptake of di- and tripeptides over a period of incubation (25 min) was considerably higher than that of FAs.

Most of the literature reports on the biosynthesis of HA have been performed at small or large scale using yeast extract as a nitrogen source. However, yeast extract has a high content of nucleic acids and RNA, which may restrict its use given the number of steps involved in the HA recovery and purification process (Von Der Haar, 2007). In addition, in the last 10 years, pharmaceutical and cosmetic companies have required the use of non-animal or microbial components in culture media for safety reasons. Soy peptones (SPs) are plant resources that meet the amino acid requirements of the genus *Streptococcus* and are a promising nitrogen source for HA production.

Pires et al. (2010 b) reported the microbial production of HA from cultures of *Streptococcus zooepidemicus* ATCC 39920 in a culture medium composed of agricultural derivatives at a G/N ratio of 7.8. The cultivations were carried out at 37°C in 250 mL Erlenmeyer flasks with reciprocal shaking at 150 rpm over 24 h. Hydrolyszed soy protein concentrate was found to be a promising nitrogen source for the production of HA (0.17 g L^{-1}) with low molar mass ($2.09x10^3$ Da).

Different plant peptones were tested by Benedini and Santana (2013), proving the effectiveness of SPs for cultures of *Streptococcus zooepidemicus* ATCC 39920 in Petri plates (inoculum) and in submerged cultivations carried out in Erlenmeyer flasks at a G/N ratio of 7.8. According to their results, BHI (37 g L^{-1}) and 5% v/v sheep blood in the solid culture

medium used for the seed culture were replaced by 67 g L^{-1} soy peptone, and HA production (0.30 g L^{-1}) and molar mass ($3.6x10^6$ Da) were maintained.

Even though the nitrogen source is known to affect HA production, studies on the uptake of amino acids by *S. zooepidemicus* are scarce. Furthermore, both yeast extract and SPs are complex nitrogen sources with varying amino acid profiles and FA and TA contents and ratios.

Given the benefits of SPs as a plant resource as well as their potential for the production of HA, the present work aimed to compare the effects of three commercial SP preparations (P1, P2 and P3) with different $FATA^{-1}$ ratios on the cultivation of *Streptococcus zooepidemicus* ATCC 39920, considering biomass and HA production as well as HA-MM. The cultivations were carried out in a 3.0 L bioreactor (working volume of 2.5 L), with forced aeration and without pH control. The SP preparations were used as complex sources of carbon and nitrogen, while glucose was used as the carbon source. The performance of the nitrogen sources was analyzed in terms of yield (biomass and HA); glucose, nitrogen and oxygen consumption; and the average HA-MM produced.

2. MATERIALS AND METHODS

2.1 Microorganism

Streptococcus equi subsp. *zooepidemicus* ATCC 39920 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was freeze-dried and kept frozen at -80°C in ampoules containing 10% glycerol.

2.2 Nitrogen sources

Three different SP preparations were purchased from different suppliers: Organotechnie (S. Courneuve, France), Kerry, Inc. (Millington Road Beloit, WI, USA) and Sigma-Aldrich Co. (Saint Louis, MO, USA), classified as P1, P2 and P3, respectively. The total protein and peptide (TPP) concentration was determined by the bicinchoninic

acidmethod (BCA) (Pierce Chemical Company/Thermo Scientific, Rockford, IL). The BCA assay, first described by Smith et al (1985), depends on the conversion of Cu^{2+} to Cu^{+}. Using the biuret method, Cu^{+2} reacts with the amine group of amino acids in protein chains, and the resulting Cu^{+} is then detected through its reaction with BCA. *Kjeldahl* nitrogen was calculated by dividing the TPP concentration by the factor 6.25, as recommended by Anvisa No. 41. The P3 preparation was centrifuged twice to precipitate insoluble components prior to quantifying *Kjeldahl* nitrogen.

2.3 Inoculum

An ampoule containing *Streptococcus equi* subsp. *zooepidemicus* ATCC 39920 was streaked onto Petri dishes containing SP (67 g L^{-1}) and bacteriological agar (Benedini and Santana, 2013). The plates were incubated at 37°C for 24 h and the colonies were then transferred into Erlenmeyer flasks (50 mL) containing sterile culture medium (25 mL) at 37°C with constant stirring at 150 rpm for 12 hours. Subsequently, the first inoculum (25 mL) was added to 225 mL of sterile culture medium, followed by incubation under the same culturing conditions for 6 hours. The culture medium used for the inocula and cultivations was composed of glucose (25 g L^{-1}) and SPs (P1, P2 or P3), at a glucose/nitrogen (G/N) ratio of 4.3.

2.4 Culture conditions

Each inoculum was transferred to a 3.0 L-bioreactor (New Brunswick Scientific Co. Inc., Edison, NJ, USA) at 10% (v/v). The temperature and agitation rate were maintained at 37°C and 250 rpm, respectively, with continuous air supply at 5 L min^{-1} and without pH control for 24 h.

Biomass and HA production and the glucose and nitrogen concentrations were determined in samples withdrawn at two-hour intervals. The average HA-MM was

determined using the final culture sample. Analyzes were carried out in triplicate, and Tukey's test was applied with 5% probability ($p < 0.05$) to compare the averages.

2.5 Separation and recovery of HA

Fermented broth was centrifuged at 1,318 g for 20 min. Each cell-free supernatant was treated with 1.5/1.0 v/v ethanol (ethanol/supernatant). The solution was cooled to 4°C for 1 hour and then centrifuged to precipitate HA. Each precipitate was then dissolved in a 0.15 mol L^{-1} NaCl solution. The precipitation process, including ethanol treatment and resuspension with NaCl, was performed twice more for HA quantification.

2.6 Analytical methods

2.6.1 Biomass

The biomass concentration was determined using the gravimetric method. During cultivation, samples containing 10 mL of the fermentation broth were centrifuged in previously dried and weighed tubes. After centrifugation, each precipitate containing the biomass was washed (with deionized water) and centrifuged three times. After the last centrifugation, the precipitate was dried and weighed.

2.6.2 Glucose

The concentration of glucose in the samples was determined using an enzymatic Glucose Bio liquid kit (Laborclin Ltd., Pinhais, Paraná, Brazil).

2.6.3 HA concentration and molar mass

The turbidimetric method (Chen and Wang, 2009) was applied to determine the concentration of HA after purification (Item 2.5). Sodium hyaluronate (Hylumed ™) from Genzyme Corporation (Cambridge, MA, USA) was used as a standard. A standard calibration curve was constructed from reactions of the HA standard with a CTAB solution, followed by spectrophotometer measurement at 400 nm.

The HA-MM weight average was determined by size exclusion chromatography (Shimadzu Corporation, Kyoto, Japan). Samples of purified HA were filtered through 0.2-µm-pore-size membranes (Sartorius, Goettingen, Germany). The filtered samples (20 µL) were injected onto a Polysep-GFC-P (7.8 mm x 35 mm) column (Phenomenex, Torrance, CA, USA), assembled in series with a Polysep-GFC-P6000 (7.8 mm x 300 mm) gel filtration column (Phenomenex, Torrance, CA, USA) and a refractive index detector (Shimadzu Corporation, Kyoto, Japan). The mobile phase was composed of $NaNO_3$ (0.1 M), pumped at a flow rate of 1.0 mL min^{-1} (Chong and Nielsen, 2003). A calibration curve was constructed using 50, 150, 250, 500 and 1000 kDa HA-MM standards.

3. RESULTS AND DISCUSSION

3.1. Aminoacid profiles of the soy peptones

The aminograms of the SP preparations in Table (1) show that P1, P2 and P3 contain the 12 essential amino acids identified by Armstrong et al. (1997) as required for *Streptococcus equi zooepidemicus* ATCC 35246 growth in a chemically defined medium: arginine, cysteine, glutamine, histidine, isoleucine, lysine, leucine, methionine, tyrosine, phenylalanine, tryptophan and valine.

Table 1–Aminograms including the total (TA) and free (FA) amino acids present in P1, P2 and P3.

Amino acids	P1			P2			P3		
	TA[a]	FA[a]	FA TA^{-1}	TA[a]	FA[a]	FA TA^{-1}	TA[a]	FA[a]	FA TA^{-1}
Alanine	2.50	1.00	0.40	2.30	0.31	0.13	2.20	0.09	0.04
Arginine	3.40	1.30	0.38	3.70	0.89	0.24	3.50	0.33	0.09
Aspartate	5.20	0.50	0.09	0.74	0.51	0.69	--	0.07	--
Cysteine	--	0.10	--	0.10	0.02	0.20	0.50	0.01	0.02
Glutamine	0.70	0.40	0.57	--	0.33	--	--	0.03	--
Glutamate	12.60	4.40	0.35	12.30	0.53	0.04	--	0.15	--
Glycine	2.20	0.30	0.13	2.20	0.28	0.13	2.10	0.09	0.04
Histidine	0.70	0.40	0.57	1.30	0.13	0.10	1.30	0.05	0.04
Isoleucine	2.30	0.80	0.35	2.00	0.03	0.02	2.20	0.02	0.01
Leucine	3.40	1.70	0.50	3.80	0.81	0.21	3.80	0.30	0.08
Lysine	3.70	0.80	0.21	3.90	0.68	0.17	3.10	0.23	0.07
Methionine	--	0.30	--	0.50	0.03	0.06	0.50	0.09	0.18
Phenylalanine	2.20	1.10	0,50	2.60	0.24	0.09	2.50	0.12	0.05
Proline	2.30	0.20	0.09	2.90	--	--	2.50	0.01	0.00
Serine	1.25	0.55	0.44	3.20	0.55	0.17	2.50	0.11	0.04
Threonine	2.00	0.60	0.30	2.20	0.25	0.11	2.10	0.06	0.03
Tryptophan	--	0.30	--	--	0.21	--	--	0.06	--
Tyrosine	1.20	0.50	0.41	1.80	--	--	2.00	0.05	0.03
Valine	2.50	1.00	0.40	2.20	0.10	0.05	2.20	0.09	0.04
Total	48.15	16.25	0.34	47.74	5.90	0.12	33.00	1.96	0.06

P1 - Soy peptone E-110, Organotechnie SAS (La Courneuve, France)

P2 - Hy-Soy, Kerry Inc. (Millington Road Beloit, WI, USA)

P3 - Peptone Hy-Soy® T (P6463), Sigma-Aldrich Co (Saint Louis, MO, USA)

a - (g 100 g^{-1})

As they are complex nitrogen sources, the SPs contain these amino acids in free and peptide forms at different concentrations. Minor differences in the P1 and P2 profiles were observed; however, P3 contains little glutamine (Table 1). In P1, glutamine was found in the FA (0.40 g 100 g^{-1}) and TA (0.70 g 100 g^{-1}) fractions, while it was found in only the FA fraction in P3 (0.03 g.100 g^{-1}). In *Streptococcus,* glutamine can be synthesized from glutamate and ammonium by glutamine synthetase (Kloosterman et al, 2006). The glutamate content in the TA fraction was similar in P1 and P2 but was absent in P3; conversely, glutamate was most abundant in P1 (4.40 g 100 g^{-1}) as a FA, followed by P2 (0.53 g 100 g^{-1}) and P3 (0.15 g 100 g^{-1}).

According to Armstrong et al. (1997), among the twelve essential amino acids, approximately 50% of the nitrogen consumed is from glutamine, which is used in the synthesis of UDP-GlcNAc, one of the precursors of the HA disaccharide unit (Chong et al., 2005).

The FA and TA amounts play an important role in elongation of the HA chain. Chen et al. (2009) demonstrated that a balance between UDP-GlcUA and UDP-GlcNAc is essential to achieve high levels of HA with high HA-MM, presumably because HA polymerization can stop or be prematurely terminated upon the depletion of UDP-GlcNAc.

The aminograms of the three SP preparations show major differences in the FAs but not the TAs. Although some amino acids contribute to biomass and HA biosynthesis, competition between FAs and peptides (TAs) for transport may influence HA production (Guirard and Snell, 1962).

3.2. Cultivations of *S. zooepidemicus*

A comparative analysis of the *S. zooepidemicus* cultivations showed that the amino acid profiles of the SPs greatly influenced the biomass yield and HA-MM (Table 2).

Table 2 – Cultivation parameters for cultures of *Streptococcus zooepidemicus* ATCC 39920 grown with forced aeration at 5 L min^{-1} and an agitation rate of 250 rpm without pH control at 37 °C over 24 h at a glucose/nitrogen ratio of 4.3 for the SP preparations P1, P2 and P3.

Parameter	P1	P2	P3
Biomass (g L^{-1})	4.51 ± 0.44[a]	2.58 ± 0.28[b]	3.11 ± 0.34[c]
HA production (g L^{-1})	0.37 ± 0.01[a]	0.31 ± 0.03[b]	0.33 ± 0.02[b]
Nitrogen consumption (g L^{-1})	1.05 ± 0.02[a]	1.21 ± 0.09[b]	2.34 ± 0.15[c]
Glucose consumption (g L^{-1})	23.62 ± 0.63[a]	13.20 ± 0.32[b]	10.85 ± 0.87[c]
Average HA-MM (10^6 Da)	0.90 ± 0.01[a]	0.64 ± 0.05[b]	0.04 ± 0.02[c]

Kinetic parameter	P1		P2		P3	
		R^2		R^2		R^2
μ_{max} (h^{-1})	0.23[a]	0.93	0.29[b]	0.95	0.36[c]	0.84
$Y_{P/S}$ (g g^{-1})	0.02[a]	0.93	0.01[a]	0.86	0.02[a]	0.94
$Y_{X/S}$ (g g^{-1})	0.18[a]	0.94	0.16[a]	0.92	0.24[b]	0.96
$Y_{P/X}$ (g g^{-1})	0.09[a]	0.95	0.10[a]	0.93	0.10[a]	0.93

Biomass,biomass produced during cultivation; HA production,hyaluronic acid produced during cultivation and purified; μ_{max}, maximum specific growth rate;$Y_{X/S}$, g of biomass produced per g of glucose consumed; $Y_{P/X}$, g of HA produced per g of biomass produced; $Y_{P/S}$, g of HA produced per g of glucose consumed; average HA-MM, average molar mass of the HA produced.

Different letters (a, b and c) indicate results that are significantly different according to Tukey tests at 95% confidence. P1, Soy peptone E-110,Organotechnie SAS (La Courneuve, France); P2, Hy-Soy,Kerry Inc. (Millington Road Beloit, WI, USA); P3, Peptone Hy-Soy® T (P6463), Sigma-Aldrich Co (Saint Louis, MO, USA).

The biomass produced followed the order: P1 > P3 > P2. In general, organic nitrogen sources provide a significant percentage of carbon for biomass production (O'Regan et al., 1994), which justifies the inverse consumption of glucose and nitrogen in the cultivations. In addition, competition for the same transport system can be reduced in the presence of peptides, since they may be more effectively transported than FAs (Guirard and Snell, 1962). Therefore, the increased nitrogen uptake observed in cultures with P3 explains their reduced glucose consumption per gram of biomass produced. Consequently, the highest $Y_{X/S}$ was observed with P3(0.24 g g^{-1}; P1 and P2 had $Y_{X/S}$ values of 0.18 g g^{-1} and 0.16 g g^{-1},

respectively), since glucose was considered the only substrate. HA production was slightly higher with P1 and was statistically equal with P2 and P3. The $Y_{P/S}$ and $Y_{P/X}$ yields were statistically similar for all three SPs (Table 2). According to Jagannath and Ramachandran (2010), the higher availability of complex nitrogen that is easily catabolizable favors biomass and leads to HA with low HA-MM. Therefore, an inverse correlation between the maximum specific growth rate (μ_{max}) and HA-MM was observed.

Fig. (1) shows the performance of the fermentations in terms of the μ_{max}, FA TA^{-1}, HA production, and average HA-MM and biomass production in culture media containing the SPs.

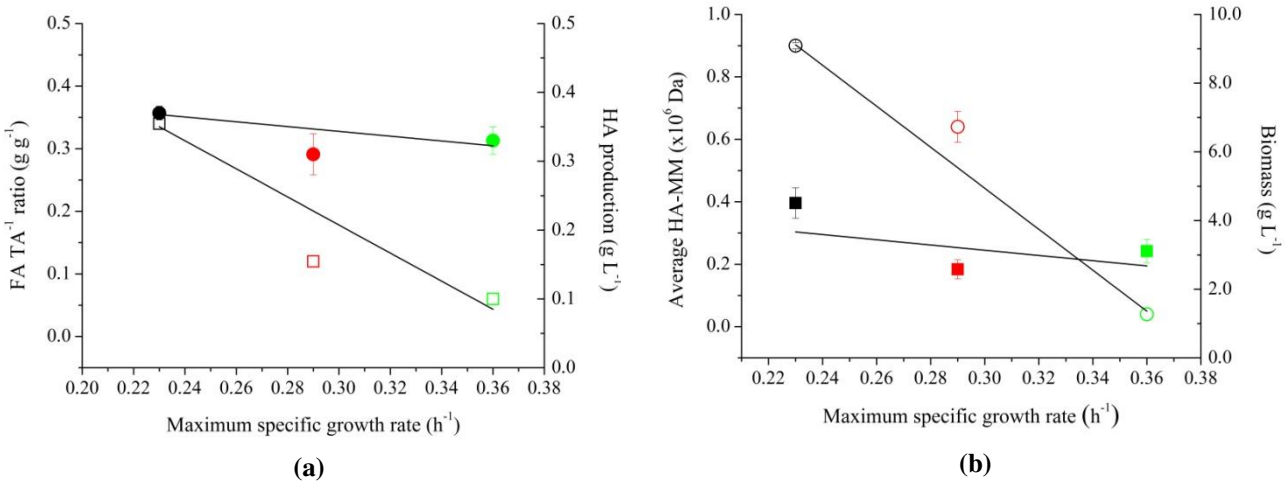

Fig. 1. Relationships among the maximum specific growth rate (μ_{max}), the free to total amino acid ratio (FA TA^{-1}) (□) and HA production (●) (a); average HA molar mass (HA-MM) (○) and biomass production (■) (b) in culture media containing the P1 (—), P2 (—) or P3 (—) SP preparation.

The results showed that the FA TA^{-1} ratio mainly affected the μ_{max} and HA-MM. The inverse relationships among both the FA TA^{-1} ratio or HA-MM and μ_{max} show that the nitrogen uptake in the SPs controls μ_{max}. The lower uptake of FA than the peptide chains present in the SPs (TA) reduces μ_{max}, and the released energy is channeled into increasing the HA-MM. The sharp decrease in HA-MM observed with P3 is also due to a lack of glutamine

and glutamate, which causes an imbalance in HA precursors (UDP-GlcUA and UDP-GlcNAc) by reducing UDP-GlcNAc production.

Similar relationships between HA-MM and the μ_{max} were obtained by Chong et al. (2005) using yeast extract as a nitrogen source and by Armstrong and Johns (1997) using a chemically defined medium. Increased biomass production increases the concentration of HA synthase (HAS) on the cell membrane. Sheng et al. (2009) reported that high expression of HAS causes an imbalance in the proportions of HAS and HA precursors and is an important factor for HA-MM control. Fig.(2) summarizes the metabolism of *S.zooepidemicus* related to HA production as well as the effect of glutamine on the production of UDP-GlcNAc and the balance of HA precursors.

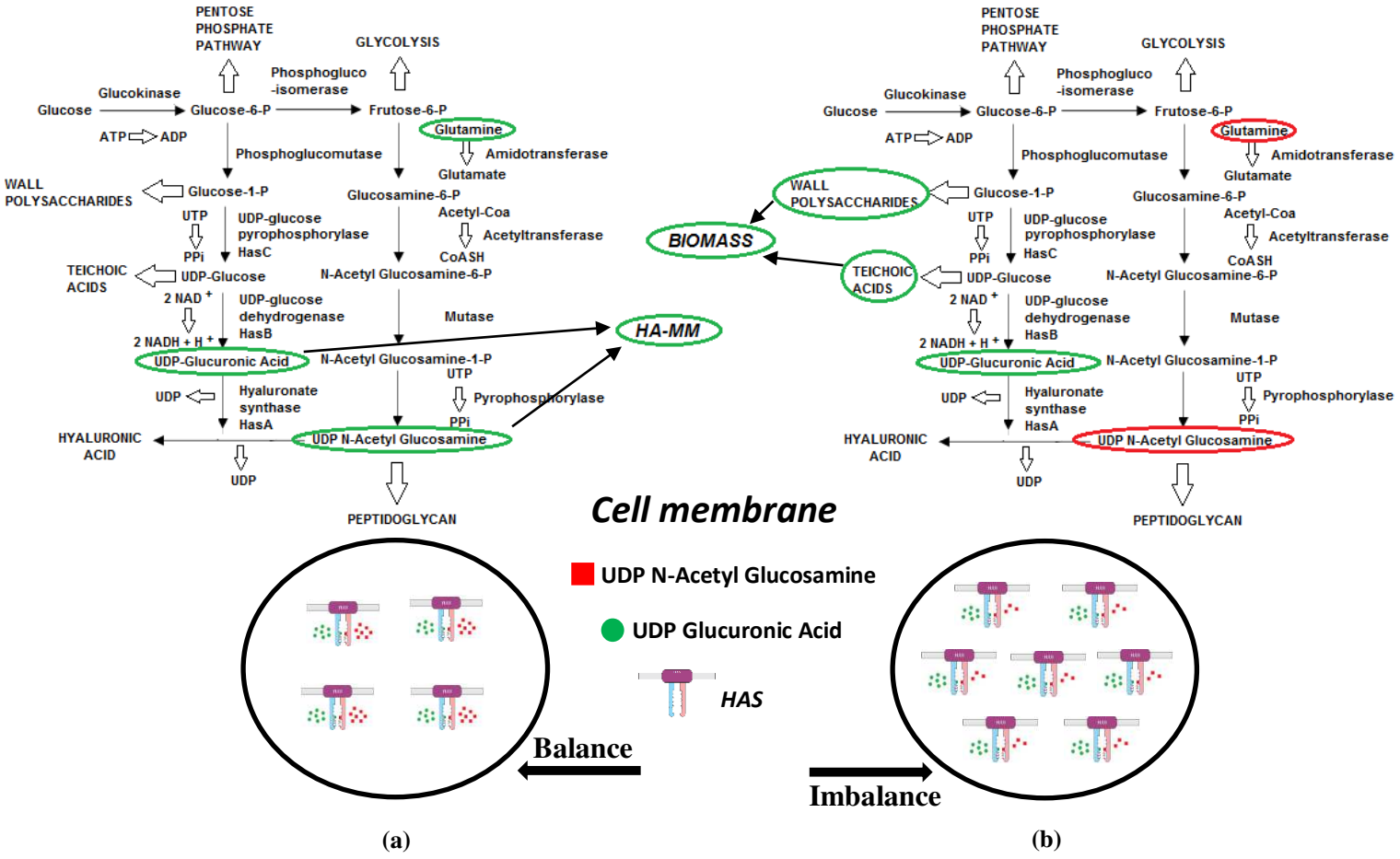

Fig 2. Biosynthetic pathway for HA production in streptococci. Increased glutamine availability (—) induces balanced UDP-N-acetylglucosamine and UDP-glucuronic acid production, which benefits HA molar mass (HA-MM) (a). Decreased glutamine availability (—) induces imbalanced UDP-N-acetyl glucosamine and UDP-glucuronic acid production, which reduces HA-MM (b). Increased (—) availability of wall polysaccharides and teichoic acids induces biomass synthesis and concentration of HA synthase (HAS) in the cell membrane. Adapted from Chong et al. (2005) and Weigel (2015).

Conditions under which the precursors (UDP-GlcUA and UDP-GlcNAc) were balanced produced HA with higher HA-MM, such as in the cultures with P1 (Fig.2a). Imbalanced conditions were observed in cultures with P3 (Fig.2b), and with P2, an intermediate state was observed.

Considering the amino acid contents of P1 and P3 (Table 1), TAs and FAs were decreased by 31% and 88%, respectively, in the latter compared to the former. The lack of glutamate and glutamine in P3 in both the FA and TA forms decreases the metabolic route responsible for the production of UDP-GlcNAc (glycolysis pathway); conversely, an increased μ_{max} induces the production of teichoic acids and other components of the bacterial cell membrane, benefiting the production of UDP-GlcUA (pentose phosphate pathway). Consequently, an increase in the μ_{max} results in an increased concentration of HAS in the cell membrane, which increases the demand for HA precursor molecules (UDP-GlcUA and UDP-GlcNAc). According to the pendulum theory described by Weigel (2015), the HA-MM is impaired by the imbalanced production of HA precursors, in this case, decreased production of UDP-GlcNAc in relation to UDP-GlcUA.

Fig.(3) shows the kinetics of biomass and HA production in relation to dissolved oxygen (D.O) during cultivations in a culture medium containing the SPs.

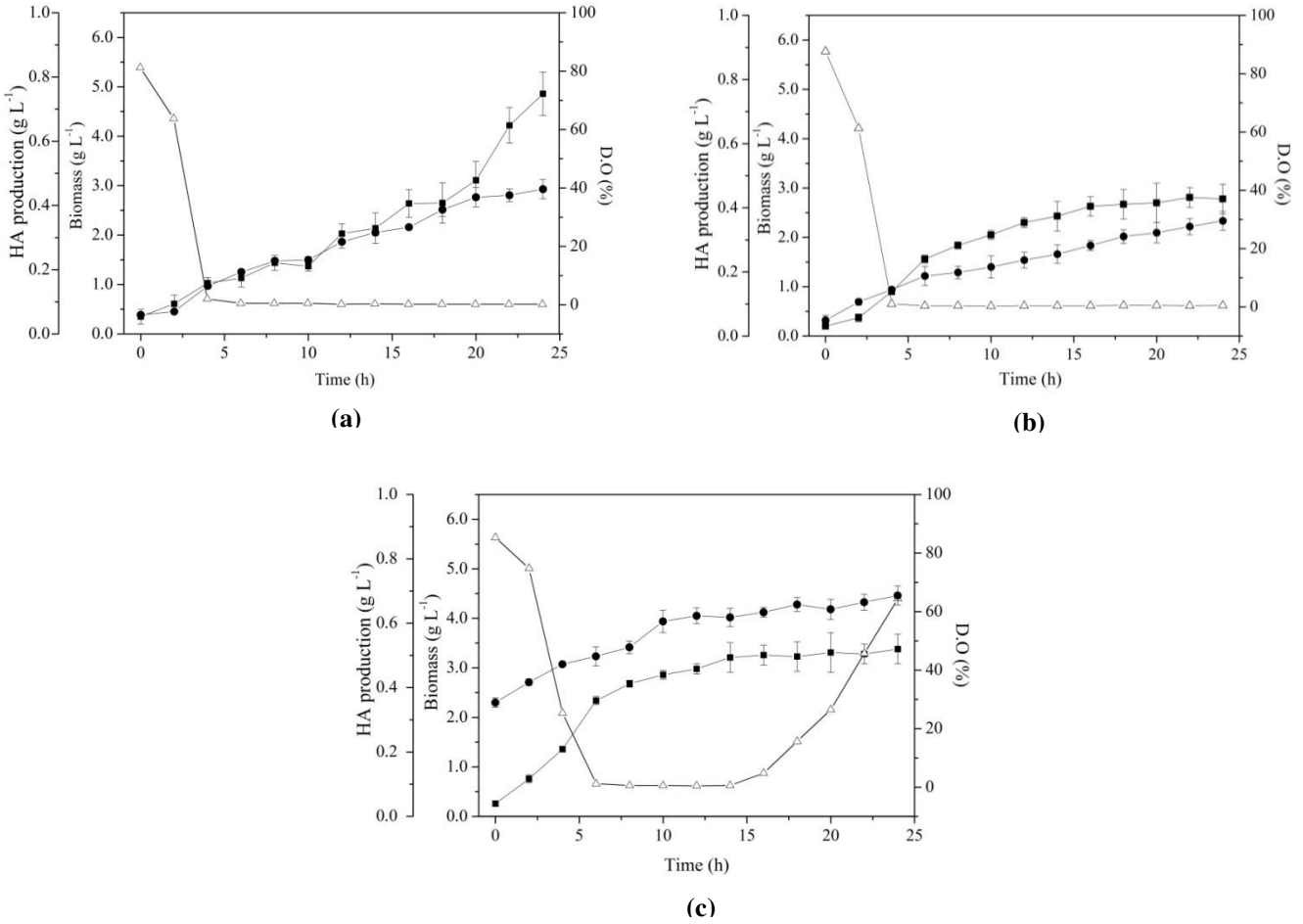

Fig. 3. Kinetic profiles of biomass (■), HA production (●) and D.O (Δ) for cultivations of *Streptococcus zooepidemicus* ATCC 39920 with the P1 (a), P2 (b), or P3 (c) soy peptone preparation in 3.0-L bioreactor with aeration and agitation rates of 5 L min^{-1} and 250 rpm, respectively, at 37°C without pH control over 24 h.

The sharp decrease in D.O at 4 h indicated the beginning of the log phase in the fermentations, after which the D.O remained constant until the end of cultivation (Fig. 3a and 3b). With P1, the biomass and HA production curves reflect the effects of the FA TA^{-1} on the μ_{max} and balance between biomass and HA production (Fig. 3a).

The presence of a stationary phase during biomass production and the increasing D.O rate after 14 hours of cultivation with P3 may indicate viability losses (Fig. 3c) that could be caused by the consumption of reduced amounts of FAs that is easily catabolized as well as the

absence of glutamine and glutamate (Table 1). The concentrations of these amino acids likely became insufficient for the continuous synthesis of biomass and elongation of HA chains.

3.3 Comparative analysis of the cultivations

Initially, a reduced FA TA^{-1} ratio benefited biomass synthesis by redirecting carbon uptake from glucose to the nitrogen source (SP). With an increased μ_{max}, the production of HAS in the cell membrane increased (Armstrong and Johns 1997), which consequently reduced the HA-MM due to changes in the ratio of HAS and HA precursor sugars (UDP-GlcUA and UDP-GlcNAc). Low initial concentrations of glutamine and glutamate were responsible for decreasing glycolysis and UDP-GlcNAc production, which imbalanced HA precursor production, reducing the HA-MM.

The higher μ_{max} that resulted from cultivation with P3 reflects the efficiency of peptide transport, as described by Guirard and Snell (1962). Therefore, because they influence the distribution of carbon and nitrogen in the metabolism of *Streptococcus* as well as the balance between UDP-GlcUA and UDP-GlcNAc for HA production, the FA concentration and FA TA^{-1} ratio of the nitrogen source are determinant factors for biomass production and HA biosynthesis.

4. CONCLUSION

The FA concentration and the FA TA^{-1} as well as the presence of glutamine and/or glutamate in SP are determinants for HA-MM control in cultivations of *Streptococcus zooepidemicus* ATCC 39920 and can be adjusted to meet the demands of specific medical applications.

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Artigo 2

CARDIOLIPIN INDUCES THE PRODUCTION OF HIGH MOLAR MASS HYALURONIC ACID FROM *STREPTOCOCCUS ZOOEPIDEMICUS* CULTIVATION

ARTIGO SUBMETIDO AO PERIÓDICO

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Cardiolipin induces the production of high molar mass hyaluronic acid from *Streptococcus zooepidemicus* cultivation

R. C. Oliveira[1] • D. S. P. Moreira[1] • M. B. Oliveira[1] • M. H. A. Santana[1*]

[1]Department of Engineering of Materials and Bioprocesses, School of Chemical Engineering, University of Campinas, Albert Einstein Avenue, 500, Campinas - SP, 13083-852.

Abstract High molar mass hyaluronic acid (HA-MM) produced by fermentation is widely used in joint treatments and regenerative medicine. However, its extrusion through the cell membrane of *S. zooepidemicus* limits the acquisition of high HA-MM. Cardiolipin (CL) is an important component of bacterial membranes, and its action is linked to cell permeability. Therefore, the effects of CL supplementation in cultivations of *S. zooepidemicus* (ATCC 39920) were evaluated in shaker flasks containing media with glucose and either yeast extract (YE) or soy peptone (SP) (Glucose/Nitrogen ratio of 4.3). In the cultivations with YE, the best result was obtained with a 30 mg L^{-1} concentration of CL, which resulted in a 35% increase in HA production and a 50% in average HA-MM. Cultivations with SP and 30 mg L^{-1} CL increased HA production three fold and average HA-MM by 33%. In conclusion, the addition of CL proved to be an advantageous strategy for the production of HA.

Keywords Hyaluronic acid - *Streptococcus zooepidemicus* - Soy peptone - Submerged fermentation – Cardiolipin

M. H. A. Santana*

1 - University of Campinas, School of Chemical Engineering, Department of Materials Engineering and Bioprocess, 500, Albert Einstein Avenue, 13083-852 - Campinas - SP - Brazil

Email: mariahelena.santana@gmail.com, Tel.: +55 19 3521 3921, fax: +55 19 3521 3890

Introduction

Hyaluronic acid (HA) is a linear polysaccharide composed of D-glucuronic acid (GlcUA) and N-acetyl-glucosamine (GlcNAc) with β-1,3 and β-1,4 glycosidic bonds. HA is a component of the extracellular tissue matrix and exists in body fluids and in human tissues at different concentrations. HA concentrations are approximately 2-3 mg ml^{-1} in human knee joints, 200 µg ml^{-1} in human eye vitreous bodies, 1 µg ml^{-1} in the aqueous humor, 0.1-18 µg ml^{-1} in human lymph fluid and typically between 10 and 100 ng ml^{-1} in the blood serum of healthy human adults [3]. The HA content in human tissues is approximately 500-2500 µg g^{-1} in cartilage, 400-500 µg g^{-1} in human skin and 1-100 µg g^{-1} in wet tissue weight for most organs, as reported by Cowman et al. [3].

Physiologically, HA in extracellular environments is constantly renewed and degraded. When the rate of HA degradation is not adequately compensated by its synthesis, changes in tissue protection, signaling and other functions are impaired, causing injuries and associated diseases. The average molar mass of HA (HA-MM) ranges from 10^4 to 10^7 Da. HA biological functions and specific applications depend on the length of its chains. HA-MM provides viscoelasticity, lubrication, structural properties and shock absorption. Moreover, cell signaling from high HA-MM has anti-inflammatory roles, while receptors for low HA-MM signal pro-inflammatory activities [1, 12].

Exogenous HA has been widely used in osteoarthritis treatments and wound healing among other diseases, including regenerative therapies. HA also has a marked niche as a vehicle for ophthalmic drugs, aesthetic procedures and cosmetic products [1].

Currently, HA produced by microbial cultivation is the most commonly used exogenous type of HA, due to its safety compared to avian derived HA. Moreover, microbial HA has physicochemical, mechanical and biological properties similar to endogenous HA [5]. High MM-HA is the HA type with the highest added value for medical applications.

Strains of *Streptococcus* produce HA to protect against environmental stresses [6]. During cultivation, HA is extruded through the cell membrane, forming a thick layer around cells that diffuses into the medium. Although HA biosynthesis is associated with biomass growth, there is competition between both biosynthesis and biomass growth for energy during cultivation [7]. *Streptococcus zooepidemicus*, the commercial and the most studied species, produces polydisperse HA-MM with 10^4 to 10^6 Da fractions depending on cultivation conditions. Various strategies have been used to enhance the HA-MM, such as the addition of lysozyme [13], incubation conditions [14], different nitrogen sources [10], genetic engineering approaches [20] and strengthening HA flux, particularly by improvements the UDP-acetylglucosamine precursor [4].

The pioneering work of Markovitz et al. [15] showed that the hyaluronate synthase (HAS) present in the membrane of streptococcus combines several functions that ultimately lead to the synthesis and translocation of the HA chain. First, HAS specifically binds the precursors UDP-GlcA and UDP-GlcNAc. Second, HAS catalyzes glycosyl transferase reactions forming β-1,4 and β-1,3 linkages between GlcA and GlcNAc residues, and, finally, it helps to form the pore responsible secreting the growing polymer [9]. *S. zooepidemicus* requires cardiolipin (CL) for maximum activity. In particular, HAS requires approximately 16 molecules of CL to become active [18].

CL is a phospholipid with a glycerol group esterified with two phosphatidylglyceride backbone fragments, and the dimeric structure composed of four acyl chains provides CL with a highly specific conical structure [16]. Weigel [19] proposed that CL lipid biding sites interact with the membrane lipid bilayer of streptococcus and the hydrophobic portions of the HA molecule. The acidic groups of CL interact with HAS and the hydrophilic sites of HA; thus, CL and HAS create pores in the membrane through which HA chains are extruded.

Based on the important roles of CL on the cell membrane, we studied the influence of CL supplementation in culture media for HA production by *S. zooepidemicus* (ATCC 39920). The culture media was composed of glucose and either yeast extract (YE) or soy peptone (SP) as a nitrogen source. In the first case, CL concentrations of 10, 20 and 30 mg L^{-1} were added to the culture medium composed of YE, while cultivations with CL at 30 mg L^{-1} were performed in culture medium with SP. The cultivations were carried out in a shaker flask with natural aeration, and the results were analyzed by biomass and HA production, $Y_{P/X}$ yield, average HA-MM content and its distribution.

Materials and methods

Microorganism

An ampoule of the microorganism *Streptococcus equi*, subsp. *zooepidemicus* (ATCC 39920), frozen at -80°C with 10% glycerol was used in all cultivations. The microorganism was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Nitrogen sources

Two different types of nitrogen sources, YE (Sigma-Aldrich Co, Saint Louis, MO, USA) and SP (Hy-Soy Kerry Inc., Beloit, WI, USA), were used in the cultivations. The Kjeldhal nitrogen in YE and SP was determined by the Kjeldahl method (ACS, 2006), (data not shown). Table (1) shows the information related to free and total amino acids present in the nitrogen sources used.

Table 1 Aminograms composed of total (TA) and free (FA) amino acids in yeast extract and soy peptone used in cultivations of *Streptococcus zooepidemicus* (ATCC 39920).

Amino acids	Yeast extract[a]			Soy peptone[b]		
	TA (g 100 g^{-1})	FA (g 100 g^{-1})	FA/TA (g g^{-1})	TA (g 100 g^{-1})	FA (g 100 g^{-1})	FA/TA (g g^{-1})
Alanine	4.1	3.8	0.9	2.3	0.3	0.1
Arginine	2.8	1.5	0.5	3.7	0.9	0.2
Aspartate	6.0	1.8	0.3	0.7	0.5	0.7
Cysteine	0.6	0.1	0.2	0.1	--	--
Glutamate	11.0	6.8	0.6	12.3	0.5	0.0
Glycine	2.5	1.3	0.5	2.2	0.3	0.1
Histidine	1.1	0.6	0.5	1.3	0.1	0.1
Isoleucine	2.6	2.2	0.8	2.0	--	--
Leucine	3.7	3.6	1.0	3.8	0.8	0.2
Lysine	4.2	1.8	0.4	3.9	0.7	0.2
Methionine	0.8	0.7	0.9	0.5	--	--
Phenylalanine	2.5	2.1	0.8	2.6	0.2	0.1
Proline	2.2	1.1	0.5	2.9	--	--
Serine	2.7	1.9	0.7	3.2	0.5	0.2
Threonine	2.6	1.7	0.7	2.2	0.2	0.1
Tryptophan	0.8	0.6	0.8	--	0.2	--
Tyrosine	0.8	0.8	1.0	1.8	--	--
Valine	3.1	2.5	0.8	2.2	0.1	0.0
Total	54.1	34.9	0.6	47.7	5.3	0.1

a - Yeast extract (Sigma-Aldrich Co)

b - Soy peptone (Hy-Soy Kerry Inc)

Inoculum and cultivation

An ampoule containing the culture of *Streptococcus equi*, subsp. *zooepidemicus* (ATCC 39920), was thawed and seeded in Petri dishes containing agar and BHI (Brain and Heart Infusion) (37 g L^{-1}) supplemented with 5% (v/v) defibrinated sheep blood (Biotério Boa Vista, SP, Brazil) in the cultivations with YE. Petri dishes containing agar and SP (67 g L^{-1}) [2] were used in the cultivations with SP. The Petri dishes were incubated at 37°C for 24 hours.

The colonies were transferred to Erlenmeyer flasks (50 mL) containing 25 mL of sterile culture medium for inoculum preparation, and the culture media was composed of either glucose (25 g L^{-1}) and YE or glucose (25 g L^{-1}) and SP. A glucose/nitrogen ratio of 4.3 was used in all cultivations. Flasks containing the inoculum were incubated in a shaker at 150 rpm and 37°C for 12 hours.

The inoculum was then transferred at 10% v/v to Erlenmeyer flasks containing the same concentrations of either glucose and YE or glucose and SP. The flasks were incubated at 150 rpm and 37°C for 24 hours. Analyses were performed in triplicate at the start and end points of each cultivation.

Cultivations with YE were performed first with CL concentrations of 10.0, 20.0 and 30.0 mg L^{-1}. In the second part of the study, cultivation with SP was performed with a CL concentration of 30.0 mg L^{-1}. Control cultivations without CL were performed with both culture media formulations.

Separation and recovery of HA

The fermented broth was centrifuged at 1318 x g for 20 minutes. The cell-free supernatant was treated with 1.5/1.0 v/v ethanol (ethanol/supernatant). Then, the solution was cooled to 4°C for 1 hour and centrifuged to precipitate HA. The precipitate was then dissolved in 0.15 M NaCl. The precipitation process with ethanol and resuspension with NaCl solution was performed two additional times for the HA quantification assay.

Analytical methods

Biomass

Biomass was determined according to the gravimetric method, and broth samples were centrifuged in tubes with known weights. After centrifugation at 1318 x g, the precipitated biomass pellet was washed and centrifuged two additional times with deionized water. Finally, the biomass was dried and weighed for gravimetric assays.

Hyaluronic acid

The turbidimetric method was applied to determine the HA content according to a previous study by Chen et al. [5]. Sodium hyaluronate (Hylumed™) from Genzyme Corporation (Cambridge, MA, USA) was used as a standard.

Hyaluronic acid molar mass

The average HA-MM was determined by size exclusion chromatography using a Shimadzu chromatography system (Shimadzu Corporation, Kyoto, Japan) connected to a column guard (Polysep-GFC-P, 7.8 mm x 35 mm; Phenomenex, Torrance, CA, USA) and a gel filtration column (Polysep-GFC-P6000, 7.8 mm x 300 mm; Phenomenex, Torrance, CA, USA). The peak profile was monitored with a Shimadzu RID-6A refractive index detector (Shimadzu Corporation, Kyoto, Japan). Twenty microliter samples were injected, and $NaNO_3$ (0.1 M) was used as the mobile phase at 1.0 mL min^{-1} and 25°C. The calibration curve used for the calculation of the HA-MM was determined using different HA-MM standards (50, 150, 250, 500 and 1000 KDa). From the calibration curve, the average HA-MM was calculated by Eq. (1) as follows:

$$\text{Log (HA-MM)} = 10.947 - 0.619 \text{ (Retention time)} \quad \text{Eq. (1)}$$

The distribution of HA-MM was calculated by Eq. (1) – (R^2 - 0.98) and the areas of the chromatographic peaks relative to 10^4, 10^5, 10^6 and 10^7 Da.

Statistical analysis

To compare the obtained media, Tukey's test was applied for all cultivations, and $p < 0.05$ was considered statistically significant (data not shown).

Results and discussion

Yeast extract cultivations

Fig. (1) shows the results of biomass and HA production in shaker flask cultures with the addition of CL at concentrations of 0, 10, 20 and 30 mg L^{-1}.

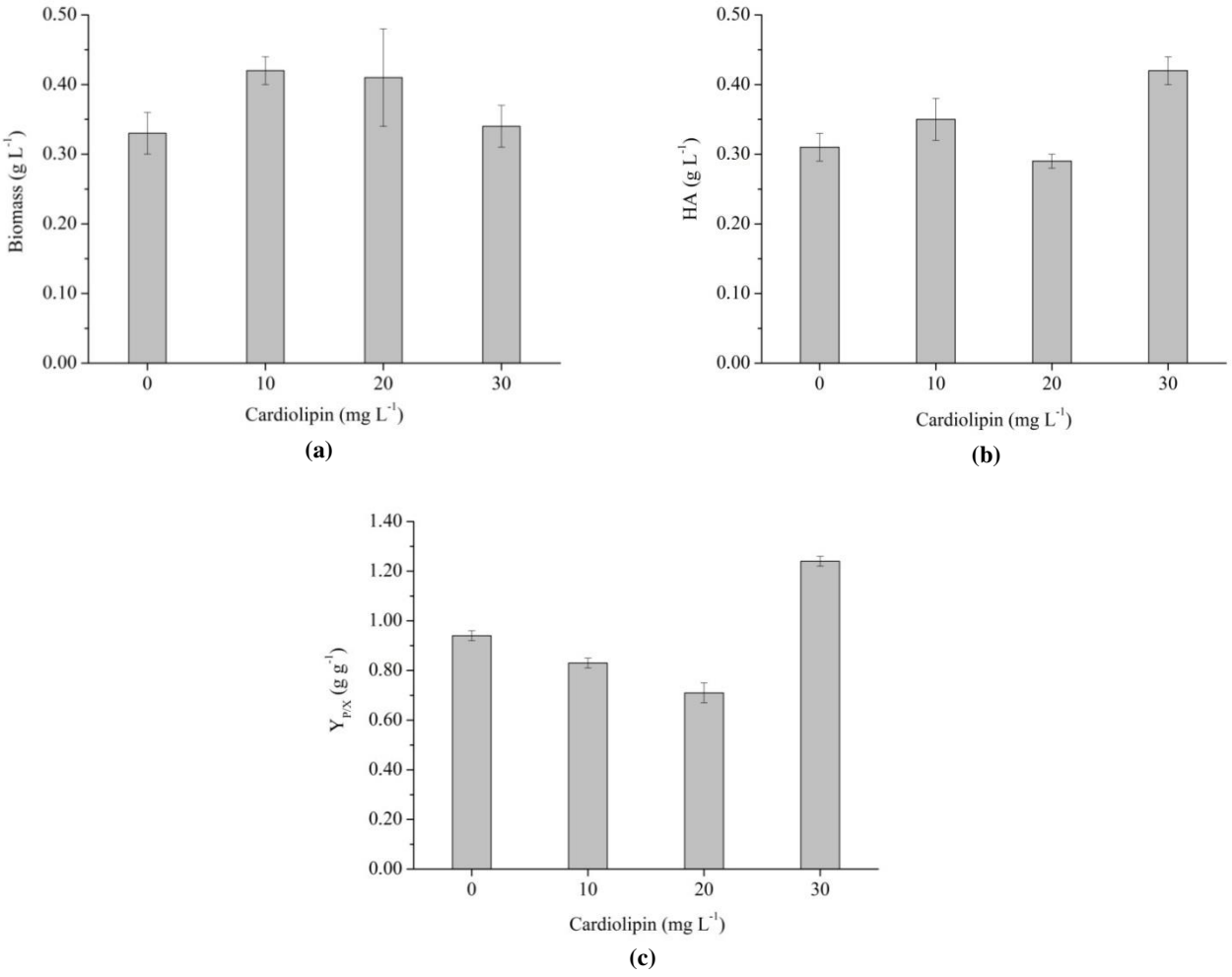

Fig. 1 Effects of the addition of cardiolipin on the production of biomass (a), HA (b) and $Y_{P/X}$ yield (c) in cultivations of *Streptococcus zooepidemicus* (ATCC 39920) in Erlenmeyer flasks, an agitation rate of 150 rpm, a controlled temperature of 37°C and culture medium composed of glucose (25 g L^{-1}) and YE at a glucose/nitrogen ratio of 4.3.

An increase of 35% in HA production was observed at 30 mg L^{-1} CL compared to the control, while the differences were not significant in cultivations with lower CL content (10 and 20 mg L^{-1}) (Fig. 1b). It is likely that the reduced CL/biomass ratio minimized the CL effects on the cell membrane at these concentrations. Regarding HA production, the increasing permeability of the cell membrane caused by the addition of CL could assist HA extrusion, increasing its production as described by Triscott and Van de Rijn [18]. As a consequence, at 30 mg L^{-1} CL, the HA yield ($Y_{P/X}$) was the highest obtained among the other results (Fig. 1c). In this case, increased HA production occurred without biomass reduction, which is in agreement with the notion that CL acts in the cell membrane only, without interference in the competition for energy between biomass and HA in the metabolic route.

Fig. (2) shows the results of average HA-MM and its distribution obtained in cultivations with YE.

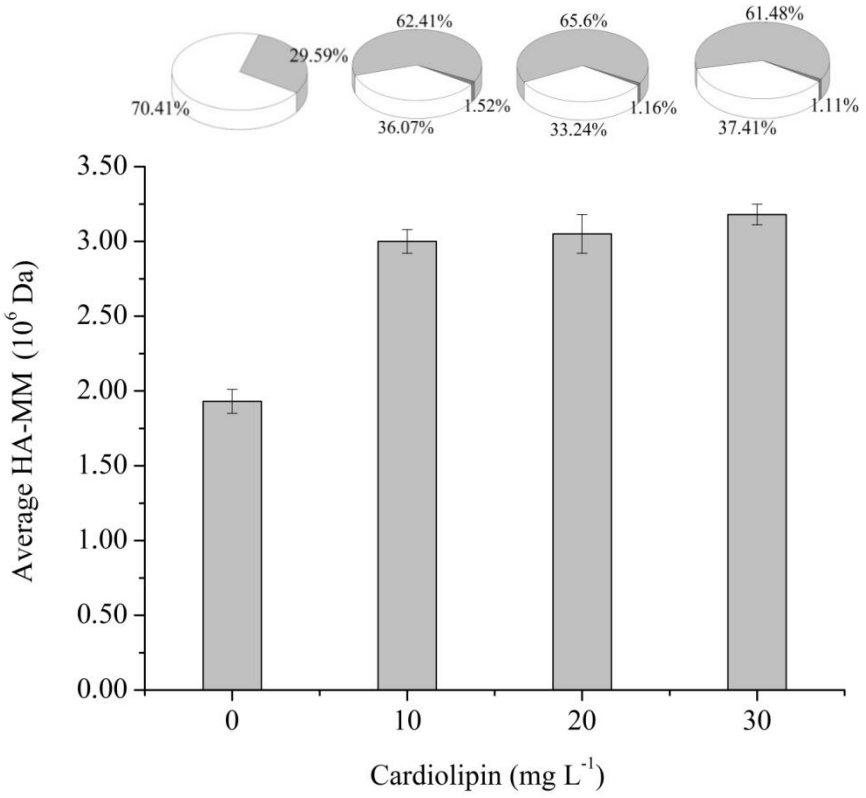

Fig. 2 Effects of cardiolipin addition on the HA average molar mass and its distribution in cultivations of *Streptococcus zooepidemicus* (ATCC 39920) in Erlenmeyer flasks, an

agitation rate of 150 rpm, a controlled temperature of 37 ºC and medium composed of glucose (25 g L^{-1}) and yeast extract at a glucose/nitrogen ratio of 4.3. HA molar mass fractions of 10^7 Da ■, 10^6 Da ■ and 10^5 Da □.

According to Fig. (2), a noticeable increase in the average HA-MM and the distribution of HA-MM with the addition of CL was observed at 10 mg L^{-1} CL, with no significant difference observed for 20 and 30 mg L^{-1} CL. However, changes in HA-MM distribution were evident with CL addition, favoring the higher HA-MM fractions.

Based on the results of HA yield ($Y_{P/X}$) and HA-MM obtained in cultivations with YE, a CL concentration of 30 mg L^{-1} was used in cultivations with SP for comparison.

Soy peptone cultivations

Fig. (3) shows the results of biomass and HA production from cultivations performed with SP. Similar to cultivations with YE Fig. (1a), there was no significant increase in biomass production with a CL concentration of 30 mg L^{-1} (Fig. 3a), reinforcing that the effects of CL occur only in the cell membrane. However, the CL effects were noticeable for HA production (Fig. 3b) and $Y_{P/X}$ yield (Fig. 3c).

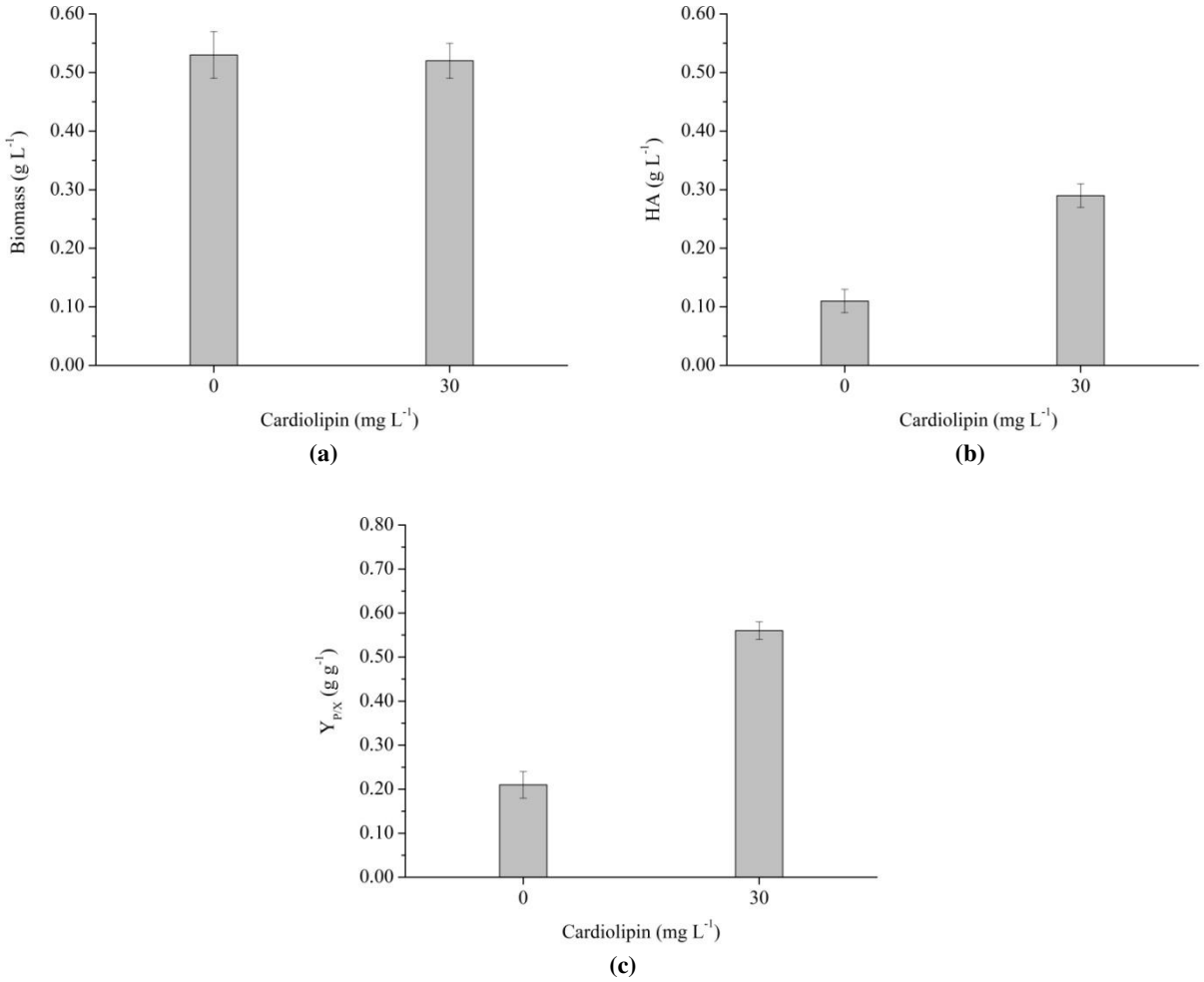

Fig. 3. Effects of the addition of cardiolipin on the production of biomass (a), HA (b) and $Y_{P/X}$ yield (c) in cultivations of *Streptococcus zooepidemicus* (ATCC 39920) Erlenmeyer flasks, an agitation rate of 150 rpm, a controlled temperature of 37 ºC and culture medium composed of glucose (25 g L^{-1}) and soy peptone with a glucose/nitrogen ratio of 4.3.

The results with SP increased HA production and yield ($Y_{P/X}$) approximately three-fold compared to the control (Fig. 3c). Fig. (4) shows the effects of CL in HA-MM produced in cultivations with SP.

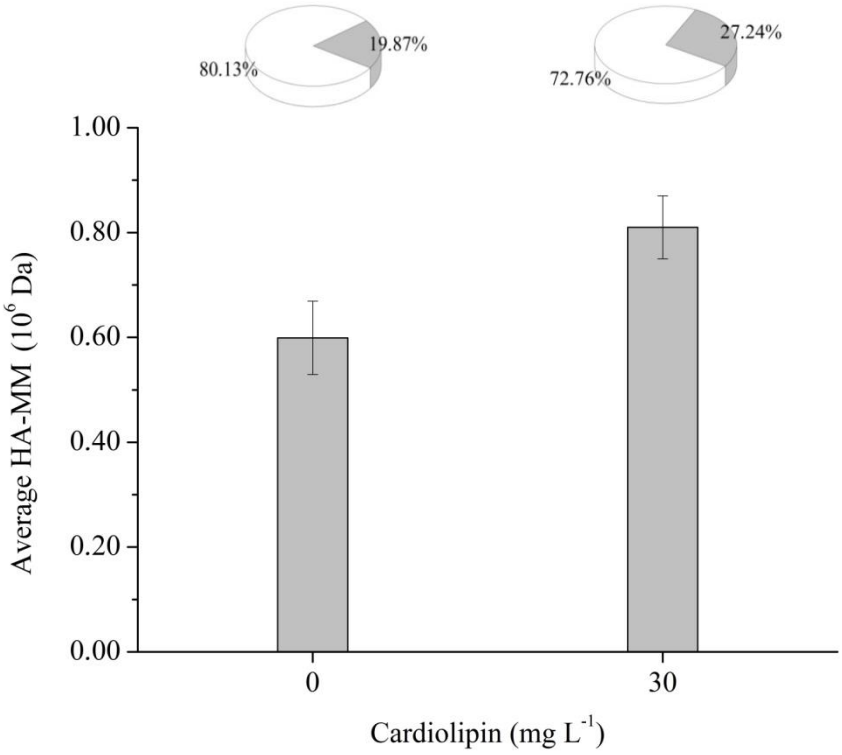

Fig. 4 Effects of the addition of cardiolipin on the HA average molar mass and its distribution in cultures of *Streptococcus zooepidemicus* (ATCC 39920) Erlenmeyer flasks, an agitation rate of 150 rpm, a controlled temperature of 37 ºC and a glucose/nitrogen ratio of 4.3 for soy peptone as the nitrogen source; molar mass distribution for 10^6 Da ■ and 10^5 Da □.

According to Fig. (4), CL increased HA-MM. This result confirms the conclusions of Weigel et al. [19], which proposed that lipids, such as CL, facilitate the extrusion of HA, thereby increasing the HA chain length. CL also increased the highest HA-MM fraction in the cultivations with SP.

These results are in accordance with the results reported by Westbrook et al. [20] in terms of the effects of CL on the cell membrane. These authors genetically engineered CL for concentration and distribution in the cell membrane of *B. subtillis* to enhance the functional expression of heterologously expressed HAS, thus improving its performance for HA production.

Comparatively, the effects of CL at 30 mg L^{-1} were effective for both HA and HA-MM production. The addition of an adequate CL concentration in the culture medium increased the permeability of the cell membrane for the extrusion of higher HA-MM. It is likely that extrusion limitations in non modified membranes could internally block HA chain elongation. The difference in absolute values seem to be linked with the capability of the nitrogen source in channeling the energy flow to the balanced production of HA precursors at the expense of biomass production, but also ensuring an effective concentration of HAS in the cell membrane to support HA production. Guirard and Snell [8] reported that the proportion of free and total amino acids in the culture medium could produce distinct results in terms of transport efficiency or that the responses to proteins or individual peptides may be different compared to free amino acids. Moreover, as complex nitrogen sources, YE and SP contain different amino acids and peptides with specific roles in microbial metabolism [11].

Therefore, from the obtained data, it can be concluded that there is an effective molecular interaction between CL added to the culture medium and the streptococcal cell membrane, thus increasing the production of HA and average HA-MM. Importantly, high average HA-MM levels were easily obtained without the need for genetic modifications. Moreover, SP has proven to be a promising nitrogen source for the production of HA, with the benefits of a plant source for producing HA for medical applications. Further studies should focus on the effects of CL on the production of HA in bioreactors with forced aeration.

Based on the results from this work and considering the market value of medical grade HA from *Streptococcus equi* with average HA-MM above $1x10^6$ Da (USD $15.90/mg - Sigma Aldrich) as well as the CL (from bovine heart) value used in the cultivations (USD $5.35/mg - Sigma Aldrich), the addition of CL to the culture media seems to be promising for the production of HA with high HA-MM.

Conclusion

The addition of cardiolipin at an adequate concentration in culture media composed of glucose and either yeast extract or soy peptone is an easy and valuable strategy for increasing the production and molar mass of hyaluronic acid produced by *Streptococcus zooepidemicus* (ATCC 39920).

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Artigo 3

OXYGEN CONTROL MODULATES MICROBIAL PRODUCTION AND MOLAR MASS OF HYALURONIC ACID SYNTHESIZED BY *STREPTOCOCCUS ZOOEPIDEMICUS*

Oxygen control modulates microbial production and molar mass of hyaluronic acid synthesized by *Streptococcus zooepidemicus*

Rhelvis de C. Oliveira[1], Matheus L. Harth[2], Antonio C. L. Horta[2], Daniela M. M. Correia[2], Teresa C. Zangirolami[2], Roberto de C. Giordano[2] and Maria H. A. Santana[1*]

[1]Department of Engineering of Materials and Bioprocesses, School of Chemical Engineering, University of Campinas, Albert Einstein Avenue, 500, Campinas - SP, 13083-852.
[2]Department of Chemical Engineering, Federal University of São Carlos, Washington Luiz Highway, 235 Km - SP 310, São Carlos - SP, 13565-905

Abstract - The aim of this research was to study the influence of oxygen on cultivation of *Streptococcus zooepidemicus* ATCC 39920 for production of high molar mass hyaluronic acid (HA-MM). The culture medium was composed of glucose and soy peptone. Submerged cultivations were carried out at controlled pH (7.0), without forced aeration or with forced aeration in uncontrolled dissolved oxygen (D.O), controlled D.O and in a mixed regime of uncontrolled and controlled D.O after 3 h cultivation. The balance between glucose and D.O determined the synthesis pathways of biomass, HA and the intermediates lactate and acetate. The excess glucose (70 g/L) and D.O control (90% saturation) along cultivation modulated HA-MM by shifting the chain size distribution to the highest fractions (from 10^4 to 10^5 and 10^6 Da). The mixed regime produced similar HA amount (2.93g/L) with the highest yield ($Y_{HA/biomass}$ - 0.24) and average HA-MM ($0.77x10^6$ Da).

Keywords – Hyaluronic acid, oxygen, *Streptococcus zooepidemicus*, soy peptone

M. H. A. Santana*

1 - University of Campinas, School of Chemical Engineering, Department of Materials Engineering and Bioprocess, 500, Albert Einstein Avenue, 13083-852 - Campinas - SP - Brazil

Email: mariahelena.santana@gmail.com, Tel.: +55 19 3521 3921, fax: +55 19 3521 3890

1. Introduction

The hyaluronic acid (HA) is a biopolymer with repetitive monomers composed by D-glucuronic acid and N-acetyl glucosamine molecules linked by β-1-3 and β-1-4 glycosidic bonds. It is present in animal tissues performing important functions such as lubrication and protection of tendons and joints (Altman et al., 2015). Due to physicochemical and biological properties, HA is widely used in facial aesthetics, ophthalmology, wound healing and orthopedy (McBride and Bard, 1979; Forrester and Balazs, 1980). The quality and function of the HA in the human body is determined by its purity and molar mass (HA-MM), which generally ranges from 10^4 to 10^7 Da (Fallacara et al., 2018).

HA can be produced by Lancefield Group C *Streptococcus* microorganisms, in particular *Streptococcus equi* sub. *equi* and *Streptococcus equi*. sub. *zooepidemicus*. The HA obtained from these microorganisms has the same properties as those of human. HA is synthesized as an extracellular capsule by *Streptococcus* strains as a virulence factor. Its disaccharide unit (D-glucoronic acid and N-acetylglicosamine) is derived from glucose-6-phosphate and fructose-6-phosphate and polymerized by the hyaluronate synthase enzyme (DeAngelis and Weigel, 1994). In addition to providing precursors for HA biosynthesis, glucose-6-phosphate and fructose-6-phosphate also provide structural constituents of the bacterial cell wall such as peptideoglycans and teichoic acids, so there is a competition for energy between biomass and HA production (Chong and Nielsen., 2003).

Since 1990, the production of microbial HA has been widely studied with glucose and yeast extract as carbon and nitrogen sources respectively. However it was also found that proteins in the yeast extract was difficult to remove due to its association with cell debris (Von Der Haar, 2007). In addition, the pharmaceutical and cosmetic industries have demanded vegetable sources in microbial culture media, to reducing the collateral problems due from protein impurities. Some studies have demonstrated the use of soy peptone as a

nitrogen source in the cultivations of *Lactobacillus acidophilus*, *Lactobacillus paracasei* ssp. *paracasei* and *Bifido bacterium lactis* (Heenan et al., 2002) and *Mycoplasma ovipneumoniae* (Patel et al., 2008). Benedini and Santana, (2013) demonstrated the efficiency of the soy peptone in inoculum preparation and HA biosynthesis from *Streptococcus zooepidemicus* ATCC 39920 in Erlenmeyer flasks, without pH control and glucose/nitrogen ratio of 7.8. Lee et al., (2009) studied the effects of sixteen non-animal nitrogen sources in cultivations of *Streptococcus sp*. KL0188 for HA production in Erlenmeyer flasks and biorreactor. Soy peptone showed the best results in terms of HA production (1.46 g/L) in cultivations in Erlenmeyer flasks.

As knowing oxygen influences HA production, however still there are few studies reporting these effects, particularly on biomass, HA production and its HA-MM. Some authors report improvements in HA production with increasing dissolved oxygen (D.O) rate, but in none of these studies the results are discussed (Huang et al., 2007; Gao et al., 2006; Chong and Nielsen, 2003).

Bacteria from *Streptococcus* genus are facultative anaerobic, but are unable to carry out oxidative phosphorylation, and thus anaerobically metabolize glucose through the Embden-Meyerhof glycolytic pathway (Wu et al., 2009). Therefore, under anaerobic conditions they produce lactate as main product, with small amounts of acetate. In aerobic conditions reductive decarboxylation of pyruvate is mediated by pyruvate dehydrogenase complex, then producing acetyl-CoA and CO_2. Acetyl-CoA would be used in the production of acetate with extra energy generation (ATP), and also in the synthesis of UDP-N-acetylglucosamine, one of the HA precursors (Chong and Nielsen, 2003).

Aerobic conditions could be beneficial for HA production due to factors as: (1) oxygen can stimulate HA biosynthesis as a protection form against its harmful effects (Cleary and Larkin, 1979; Chong and Nielsen, 2003); (2) Increasing D.O rate in the medium could

redirect part of the carbon flux to the production of acetate instead of lactate, with an extra production of ATP ($Y_{ATP/glucose}$ is 3 mol/mol), required for HA production, instead of 2 mol/mol required with lactate production (Gao et al., 2006; Chong and Nielsen, 2003). (3) Oxygen could stimulate the production of acetyl-CoA and its excess could be directed to the synthesis of HA (Wu et al., 2009).

Huang et al., (2007) used *S. zooepidemicus* ATCC 39920 and a culture medium composed by glucose (20 g/L) and yeast extract (10 g/L) in 3 L fermenter. It was found that D.O above 5 % would not result in a significant increase of HA production. However, according to Duan et al., (2009), by increasing D.O rate to values above 50% could cause disruption of the HA chain due to the generation of reactive oxygen species (ROS). Although ROS could be harmful to HA-MM, the addition of antioxidants such as tannic acid, oxalic acid, salicylic acid would reverse its effects (Im et al., 2009).

As far as we know, there are no studies in the literature reporting the effects of D.O control along cultivations. Therefore, the aim of the present work is systematically to study the influence of oxygen on HA production in a *Streptococcus zooepidemicus* ATCC 39920 in a culture medium composed of glucose and soy peptone. Three cultivations were performed with increasing oxygen flow in uncontrolled D.O (F1 < F2 < F3). The effects of D.O control were also investigated at 90% saturation along 20 hour of cultivation (F4 e F5) and in a mixed regime of uncontrolled followed by controlled D.O at 90% saturation (F6). The results were analyzed in terms of production of biomass, HA, the lactate and acetate metabolites, average HA-MM and its distribution.

2. MATERIAL AND METHODS

2.1. NITROGEN IN SOY PEPTONE

The total protein and peptide (TPP) content in soy peptone (Hy-Soy, Kerry Inc, WI, USA) was determined by bicinchoninic acid assay (BCA; Pierce Chemical Company/Thermo

Scientific, Rockford, IL). Nitrogen Kjeldahl was calculated by dividing the TPP content by a factor of 6.25, as recommended by Ordinance No.360 from Anvisa.

2.2. TOTAL CARBON IN SOY PEPTONE

The total organic carbon (TOC) concentration was determined using a TOC-L (Shimadzu, Kyoto, Japan) analyzer with a catalytic oxidation method (Sugimura and Suzuki, 1988).

2.3. MICROORGANISM

Strains of *Streptococcus equi*, subsp. *zooepidemicus* ATCC 39920 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), lyophilized and kept frozen at -80 °C in ampoules containing 10% glycerol.

2.4. INOCULUM AND FERMENTATION

The inoculum was prepared from an ampoule containing the microorganism, which has previously been streaked in Petri plates containing soy peptone (67 g/L) and bacteriological agar (Benedine and Santana, 2013). The plates were incubated at 37°C for 24 hours, colonies were transferred to an Erlenmeyer flask (50 mL) containing 25 mL culture medium kept under constant stirring at 150 rpm, 37°C for 12 hours. Then, the inoculum was added to the 225 mL sterilized culture medium in a 500 mL Erlenmeyer flask, under the same stirring and temperature for 6 hours.

The inoculum was transferred at 10% v/v to 3.0 L BioFlo III bioreactor (New Brunswick Scientific Co. Inc., Edison, NJ, USA). For cultivations with controlled D.O at 90%, the inoculum was transferred at 10% v/v to a 5 L home-made bioreactor monitored by SUPERSYS_HCDC (Horta et al., 2014). The D.O concentration in the bioreactor was controlled at 90% by a hybrid controller (PID+Heuristic) with changes in agitation rate and a mixing pure oxygen and air flow rate (GFC AALBORG).

In all cultivations pH was controlled (on/off) at 7.0 with NaOH solution (20% w/v) and the temperature at 37 ºC during 20 hours. The samples were taken every two hours with assays performed in triplicate in all cultivations.

Table (1) shows the conditions used in F1 to F6 cultivations.

Table 1 – Oxygen control and C/N ratio data used in F1to F6 cultivations.

Cultivation	Glucose (g/L)	C/N Ratio (g/g)	Agitation Rate (rpm)	Air Flow Rate (L/min)	OxygenFlow Rate (L/min)	D.O (%)	Oxygen Inlet (%)
F1	25.0	10.8	250	0.0	0.0	-	0
F2	25.0	10.8	250	5.0	0.0	-	21
F3	25.0	10.8	250	5.0	0.5	-	28
F4	25.0	10.8	250 – 900	0.0 – 5.0	0.0 – 4.0	90	-
F5	70.0	30.2	250 – 900	0.0 – 5.0	0.0 – 4.0	90	-
F6[a]	70.0	30.2	250 – 900	0.0 – 5.0	0.0 – 4.0	90	-

a – Control of D.O at 90% after 3 hours of cultivation

The culture media used in inoculum and fermentation were composed by glucose (carbon source) and soy peptone in sufficient quantities for the use of a C/N ratio 10.8 (g/g) in F1 to F4 cultivations. Except for F1 (no continuous oxygen supplying), the oxygen was provided from atmospheric air in F2 (5.0 L/min) or from atmospheric air and pure oxygen in F3 (5.0 L/min and 0.5 L/min respectively) performing 28% oxygen, and F4 with D.O controlled at 90% saturation. Agitation rate was maintained at 250 rpm in F1, F2 and F3. In F4 agitation rate was controlled by the D.O control system from 250 to 900 rpm, aeration and oxygen rate ranging from 0 to 5 L/min and 0 to 4 L/min respectively. The same control system at 90 % was used in the other two cultivations (F5 and F6), performed with initial glucose concentration at 70 g/L or C/N ratio of 30.2 (g/g), used in inoculum and fermentation stages. In F6, uncontrolled D.O was used only in the first 3 hours of cultivation.

2.5. HA SEPARATION AND PURIFICATION

The cultivation broth was centrifuged at 1,318×g for 20 min. The cell-free broth was treated with ethanol 1.5:1 v/v (ethanol/supernatant). The solution was refrigerated at 4°C for 1h for HA precipitation by centrifugation (1,318×g for 20 min). The precipitated HA was dissolved in a 0.15 mol/L NaCl solution. This process of precipitation and dissolution was repeated twice.

2.6. ANALYTICAL METHODS

BIOMASS

Biomass was determined using the gravimetric method. Broth samples were centrifuged in tubes with known weights. After centrifugation at 1318 x g, the precipitated biomass pellet was washed and centrifuged two additional times with deionized water. Finally, the biomass was dried and weighed for gravimetric assays.

GLUCOSE

Glucose concentration was determined using a commercial glucose oxidase kit (Laborlab Ltda, Guarulhos, São Paulo, Brazil).

CONCENTRATION AND AVERAGE HA-MM

The turbidimetric method was applied to determine the concentration of HA in the purified broth (Item 2.5) (Chen and Wang, 2009). The average HA-MM was determined by size exclusion chromatography in a Polysep-GFC-P 7.8mm x 35mm column guard (Phenomenex, Torrance, CA, USA) connected to a Polysep-GFC-P6000 7.8mm x 300mm column (Phenomenex, Torrance, CA, USA) and a refractive detector index (RID 6A, Shimadzu Corp, Kyoto, Japan). Mobile phase was composed by $NaNO_3$ (0.1 M) with flow rate of 1.0 ml/min. The calibration curve used in the calculation of the HA-MM was constructed from different HA-MM standards (50,150, 250, 500 e 1000 KDa). From calibration curve, the average HA-MM was calculated by Eq (1).

$$\text{Log (HA-MM)} = 10.947 - 0.619 \text{ (Retention time)} \quad \text{Eq. (1)}$$

The distribution of HA-MM was calculated by Eq. (1) – (R^2 - 0.98) and the areas of the chromatographic peaks relative to 10^4, 10^5, 10^6 and 10^7 Da.

CONCENTRATION OF METABOLITES

Samples of fermentation broth were filtered through membranes with a 0.2 μm pore size (Sartorius, Goettingen, Germany). The filtered samples (20 μL) were injected into an ion exchange HPLC (Shimadzu Corporation, Kyoto, Japan) connected to an HPX-87H fast acid column Aminex (Bio-Rad,70 Hercules, CA., USA) with dimensions of 7.8 mm x 300 mm. The mobile phase was composed of H_2SO_4 (0.004 M) pumped at a flow rate of 0.6 mL/min (Chong and Nielsen, 2003). The temperature was maintained at 65°C, and the peak profile was monitored with a Shimadzu RID-6A refractive index detector (Shimadzu Corporation, Kyoto, Japan).

CONCENTRATION OF AMINO ACIDS

Amino acid analysis in F5 cultivation required deproteinization of the samples (removal of remaining proteins and oligopeptides) with ultrafilters (Millipore, Amicon, 3kDa). For the ultrafiltration assays, 0.5 mL of each sample was centrifuged at 4°C and 8,000 rpm for 8 minutes. The amino acid analysis comprises derivatization of the centrifuged solution with PITC (phenylisothiocyanate) and separation of the amino acids in a pico-tag column (3.9mm x 300mm) with an UV detector (W486) at 254 nm connected to an HPLC (High Performance Liquid Chromatography - Waters Co). Mobile fase was composed by 1 liter of sodium acetate trihydrate (19 g/L), 0.5 mL of triethylamine, 0.2 ml EDTA (1 g/L), 64 mL acetonitrile and pH adjusted at 6.4 with acetic acid solution (High Performance Liquid Chromatography - Waters Co) .

3. RESULTS AND DISCUSSION

3.1. UNCONTROLLED OXYGEN CULTIVATIONS

Fig. (1) shows the profiles of glucose, biomass, HA and D.O obtained from cultivations with 25 g/L initial glucose, controlled pH at 7.0 and conditions of non-forced and forced aeration with atmospheric air (21% oxygen) and enriched air (28% oxygen).

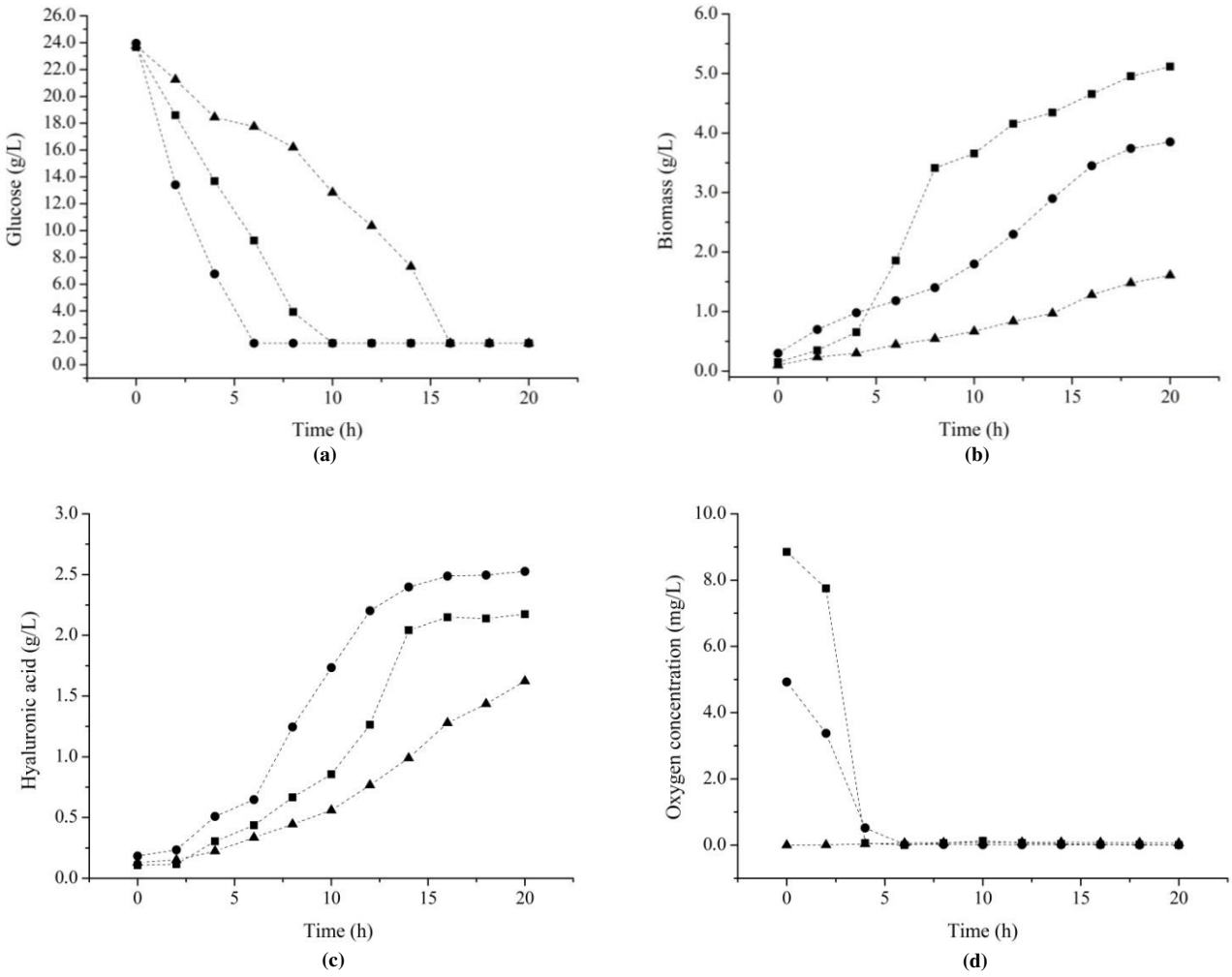

Fig. 1. Profiles of glucose (a), biomass (b), hyaluronic acid (c), oxygen concentration (d) from cultivations of *Streptococcus zooepidemicus* ATCC 39920 with non-forced and forced aeration at uncontrolled oxygen concentration. Cultivations were performed with 25 g/L glucose and soy peptone at C/N ratio of 10.8, pH control (7.0) and agitation rate of 250 rpm. F1 – non-forced aeration (▲), F2 – forced aeration at 5 L/min with atmospheric air (21%

oxygen) (●), F3- forced aeration with enriched air at 5 L/min atmospheric air and 0.5 L/min pure oxygen (28% oxygen) (■).

Fig. (2) shows the profiles of lactate and acetate concentrations, the profile and distribution of HA-MM for the culture conditions of Fig. (1).

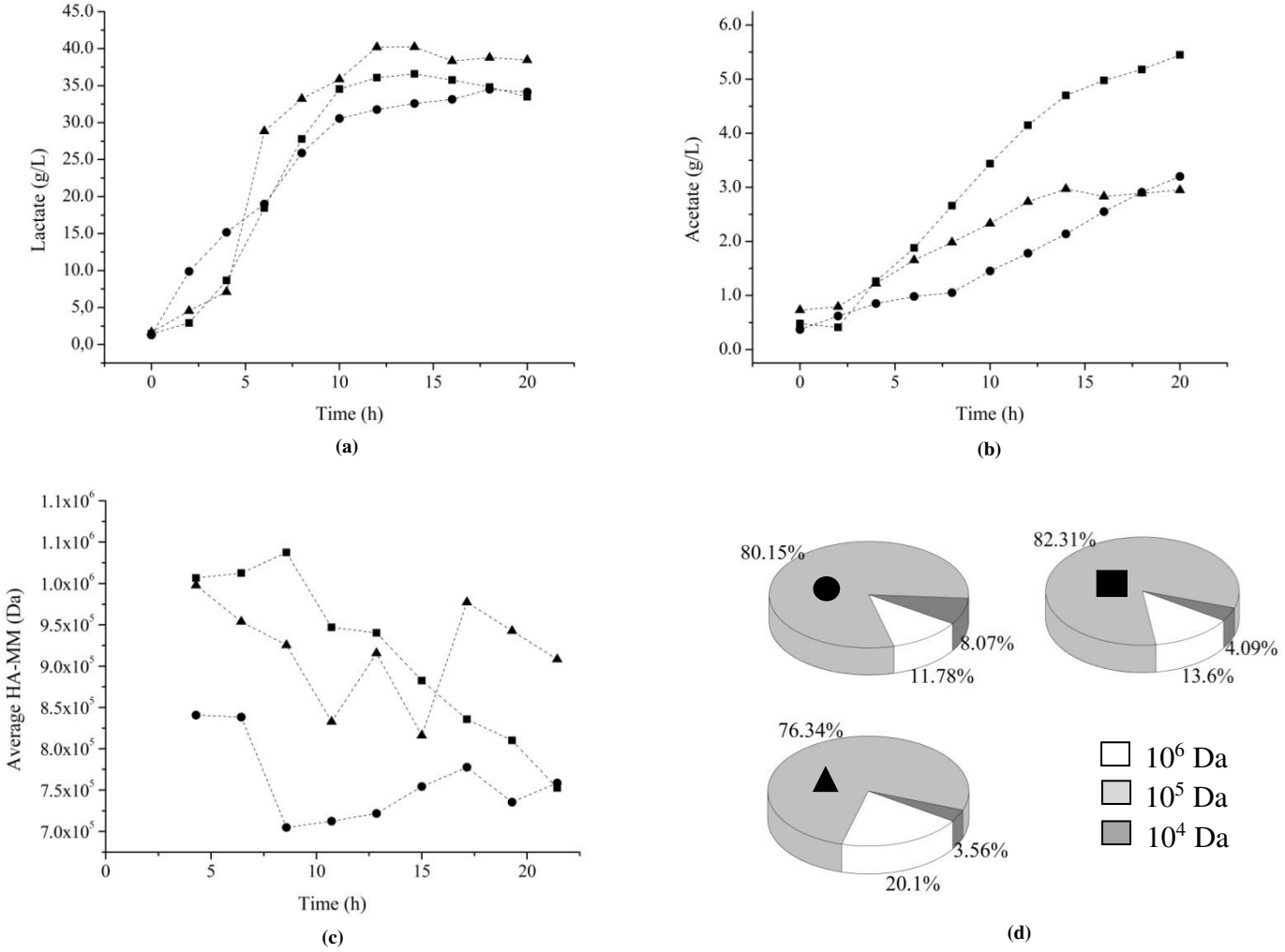

Fig. 2. Metabolites (a,b), HA average molar mass (HA-MM) (c) and molar mass distribution (d) at the end of cultivations with *Streptococcus zooepidemicus* ATCC 39920 performed with 25 g/L glucose and soy peptone at C/N ratio of 10.8, pH control at 7.0 and agitation rate of 250 rpm. F1 – non-forced aeration (▲), F2 – forced aeration at 5 L/min (atmospheric air) (●), F3 - forced aeration with enriched air at 5 L/min atmospheric air and 0.5 L/min pure oxygen (28% oxygen) (■).

The F1 profile demonstrates the facultative anaerobic capability of *Streptococcus* genus to metabolize glucose through the Embden-Meyerhof glycolytic pathway. The limitation of oxygen in F1 leaded to slower consumption of glucose and lower production of biomass and HA. Pires and Santana, (2010) observed that HA production upon limited oxygen was independent of initial glucose concentration above 5 g/L. However, the limitation of oxygen produced high average HA-MM along cultivation (Fig. 2c), with 20.1% of 10^6 Da, followed by 76.34% of 10^5 Da and 3.56% of 10^4 Da (Fig. 2d).

At reduced HA production, the energy flux is redirected to the increment of the polymer chain. In addition, limited oxygen may have been responsible for reduction of free radicals in the medium. As described by Duan et al., (2009), it may also have contributed to the higher average HA-MM obtained in F1.

It could be observed that glucose consumption rate decreased from F1 < F3 < F2, with exhaustion times of 16, 10 and 6h respectively (Fig. 1a). Biomass exponential phase was anticipated in F3 compared to F2 (Fig. 1b), in agreement with oxygen depletion (Fig. 1d). Complex culture medium such as soy peptone may contain other carbon sources, ensuring biomass biosynthesis even after glucose depletion. This justifies the behavior of biomass in the culture F3 at slower growth rate (Fig. 1b) even after total depletion of glucose in 10h (Fig. 1a). The anticipation of biomass exponential phase delayed HA production in F3 due to competition of energy fluxes between biomass and HA biosynthesis (Fig. 1b and 1c). This behavior also justifies the high HA production rate after the exponential phase of biomass in F3 (Fig. 1b and 1c).

Gao et al., (2006) concluded that biomass increased under high ATP and NADH, however HA production was inhibited at different D.O rates in cultivations with *Streptococcus zooepidemicus* H23 in 2.5 L bioreactor and culture medium containing glucose (20 g/L) and yeast extract (20 g/L) for HA biosynthesis.

Oxygen depletion rate increased from F2 < F3 (6h for F2 and 4h for F3) (Fig. 1d), in agreement with the biomass exponential phase (Fig. 1b). The forced aeration in F2 and F3 increased oxygen levels in the medium, and the oxidative phosphorylation pathway leads to Acetyl-CoA and acetic acid production, and the extra ATP generation provides the energy requirements for the synthesis of UDP-N-acetylglucosamine, one of the HA precursor (Liu *et al*., 2011). The higher oxygen consumption rate in F3 explains the higher acetate production compared to F2 (Fig. 2b) and the extra production of ATP, however biomass is benefited according to Fig. (1b). According to Fig. (2a) lactate production does not appear to have been influenced by oxygen, in agreement with Gao et al., (2006).

Similar to F1, the average HA-MM in F2 remained almost constant along cultivation, in F3 was observed reduced average HA-MM along cultivation (Fig. 2c). At the end of cultivation, HA-MM distribution showed higher fractions of 10^6 Da for F1 and similar results for F2 and F3 (Fig. 2d). The oxygen enrichment benefited HA-MM at the first hours of cultivation and the presence of ROS could be responsible for HA-MM decrease in F3 (Duan et al., 2009). Therefore, F3 cultivation could be ended between 12-15h benefiting HA-MM.

Table (2) summarizes the behavior profiles of *S. zooepidemicus* along cultivations in terms of yield parameters, maximum specific growth rate (μ_{max}) and average HA-MM.

Table 2 – Yields, maximum specific growth rate (μ_{max}) and average HA molar mass (HA-MM) determined from cultivations of *S. zooepidemicus* ATCC 39920 with non-forced and forced aeration using atmospheric (21% oxygen) and enriched air (28% oxygen).

	F1	**F2**	**F3**
$\mathbf{Y_{HA/Biomass}}$ **(g/g)**	1.04	0.69	0.41
$\boldsymbol{R^2}$	0.991	0.915	0.837
$\mathbf{Y_{Biomass/Glucose}}$ **(g/g)**	0.05	0.03	0.15
$\boldsymbol{R^2}$	0.988	0.999	0.918
$\mathbf{Y_{HA/Glucose}}$ **(g/g)**	0.04	0.02	0.03
$\boldsymbol{R^2}$	0.961	0.878	0.939
$\boldsymbol{\mu_{max}}$ **(1/h)**	0.13	0.11	0.32
$\boldsymbol{R^2}$	0.984	0.989	0.937
Average HA-MM (x10^6)	0.91	0.76	0.75
SD	0.01	0.03	0.01

$Y_{Biomass/Glucose}$– Biomass produced per glucose consumption; $Y_{HA/Biomass}$– HA production per biomass produced; $Y_{HA/Glucose}$– HA production per glucose consumption; μ_{max} – maximum specific growth rate; F1 - aeration rate of 0 L/min; F2 - aeration rate of 5 L/min (21% oxygen); F3 - aeration rate of 5 L/min and oxygen flow rate of 0.5 L/min (28% oxygen).

The cultivation at F1 condition resulted in the highest HA yield, including $Y_{HA/Biomass} > 1$. Although the lowest $Y_{HA/Biomass}$ yield, the cultivation with enriched air (F3) produced the highest μ_{max} (Table 2).

3.2. CONTROLLED OXYGEN CULTIVATIONS

The Fig.(3) shows the cultivation results with controlled D.O at 90% and glucose at 25 g/L.

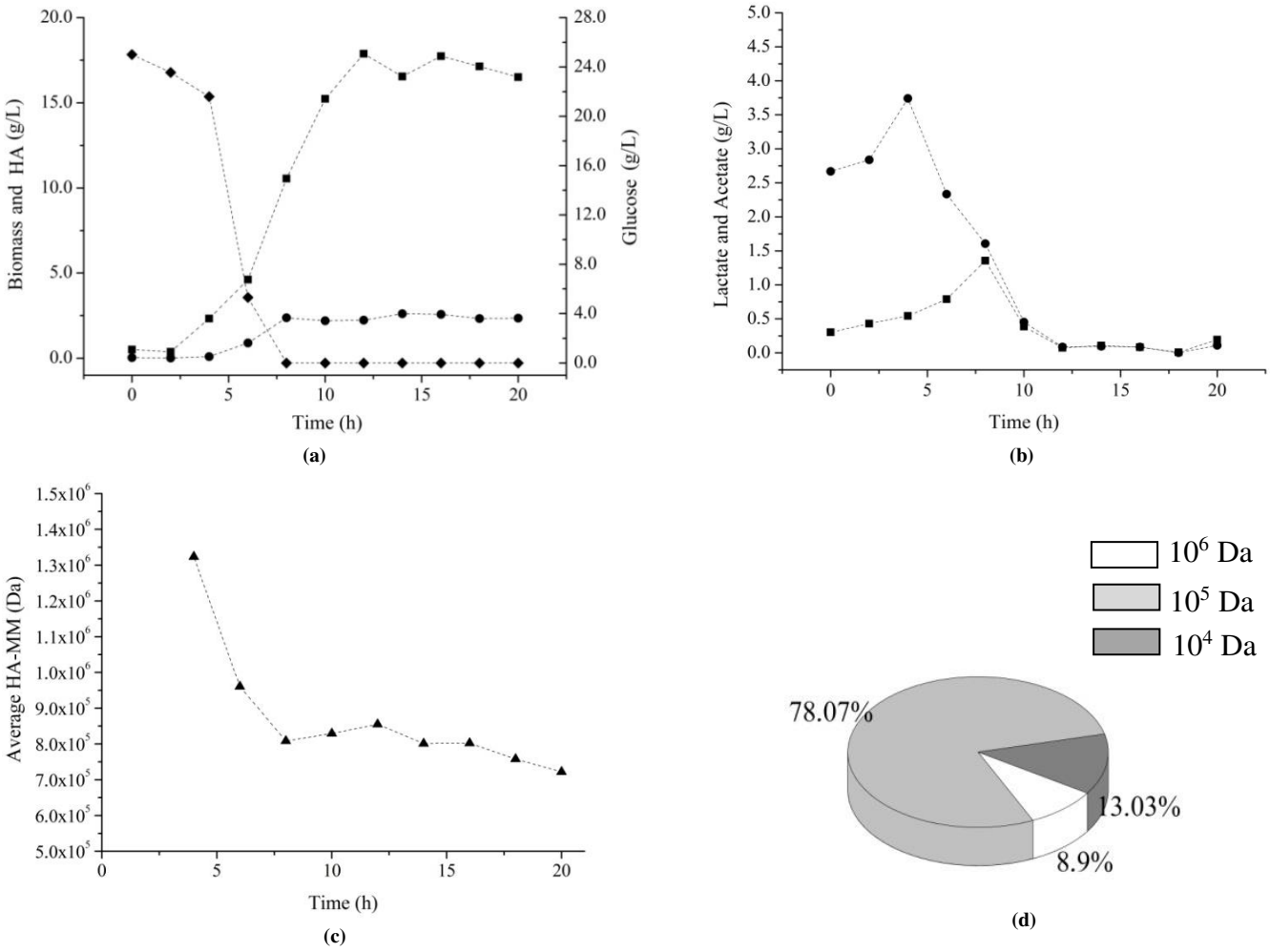

Fig. 3. Profiles of glucose (♦), biomass (■) and HA (●) (a), lactate (●) and acetate (■) (b), average HA-MM (c) and HA-MM distribution at 20h (d), from cultivations of *Streptococcus zooepidemicus* ATCC 39920 performed in F4 condition: Culture medium composed of 25g/L glucose and soy peptone at C/N ratio of 10.8, pH control at 7.0 and oxygen dissolved control at 90% saturation and temperature at 37ºC.

The effects of oxygen control along the cultivation with 25 g/L glucose can be analyzed comparing the profiles at F4 condition (Fig. 3) with the ones at the F2 and F3 conditions (Fig. 1 and 2). Glucose depletion in F4 was faster (8h) (Fig. 3a) compared with cultivation using enriched air, 10 h, (F3 condition), but slower than F2 condition, (6 h) (Fig 1a). Biomass growth in F4 followed the same glucose lag time and its production (16.51 g/L) was 3.2 and 4.3 times higher than F3 (5.12 g/L) and F2 (3.85 g/L) respectively. However, HA production in F4 reached similar level (2.35 g/L) in 20h cultivation compared to F3 (2.06 g/L) and F2 (2.43 g/L) cultures.

The concentration of metabolites (lactate and acetate) produced in F4 was reduced compared to F1, F2 and F3 conditions, indicating consumption of lactate and acetate along cultivation (Fig. 3b). The final average HA-MM in F4 ($7.2x10^5$ Da) was smaller compared to F1, F2 and F3 (Table 2). HA-MM distribution in F4 showed the fraction of 10^6 Da decreased drastically to 8.9%, increasing the lower fraction to 13.03% (10^4 Da) (Fig. 3d). In F2 and F3 these fractions were 11.78% (10^6 Da) and 8.07% (10^4 Da), 13.06% (10^6 Da) and 4.09% (10^4 Da) respectively (Fig 2d). The reduction of HA-MM is consistent with the breaking of HA provided by oxygen reactive species (ROS) generated by oxygen excess (Duan et al, 2009). Ending F4 fermentation in 10-12h approx. could prevent reduction of HA-MM.

Fig. (4) shows the relationships between the maximum specific growth rate (μ_{max}) with average HA-MM and $Y_{HA/Biomass}$ yield for F1 to F4 conditions.

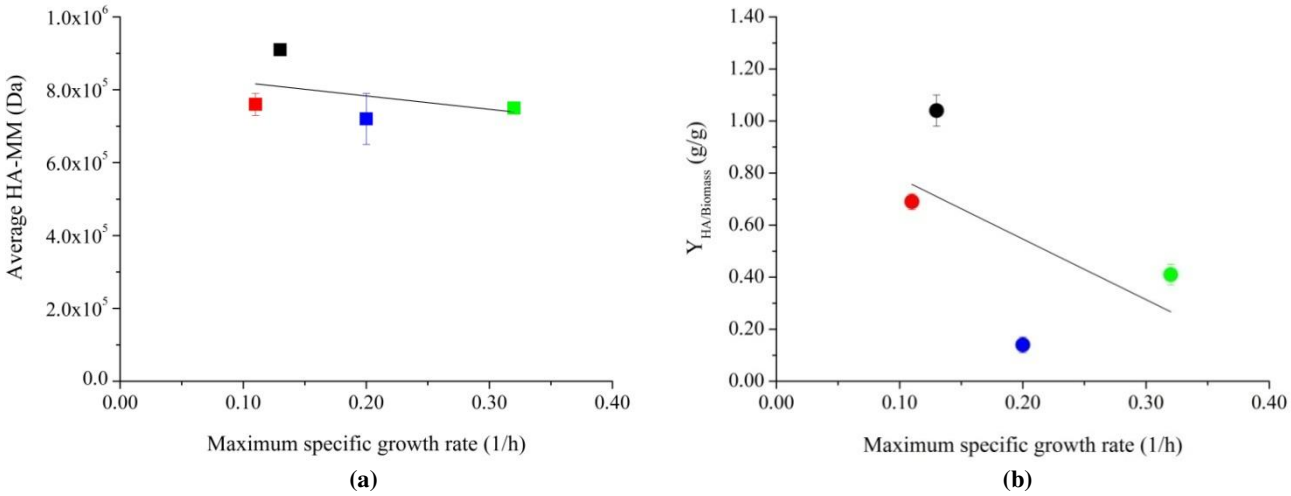

Fig. 4. Correlations between maximum specific growth rate and average HA-MM (a) and $Y_{HA/Biomass}$ yield (b), from cultivations of *Streptococcus zooepidemicus* ATCC 39920 performed with 25 g/L glucose and soy peptone at C/N ratio of 10.8, pH control at 7.0 with controlled temperature at 37 ºC during 20 hours. F1 – non-forced aeration and agitation rate at 250 rpm (■, ●), F2 – forced aeration at 5 L/min (21% oxygen) and agitation rate at 250 rpm (■, ●), F3 - forced aeration with enriched air at 5 L/min atmospheric air and 0.5 L/min pure oxygen (28% oxygen) and agitation rate at 250 rpm (■, ●) F4 – dissolved oxygen control at 90% saturation (■, ●).

Jagannath and Ramachandran, (2010) reported that with increasing μ_{max}, the average HA-MM and $Y_{HA/Biomass}$ yield are reduced due to the high expression of HAS. The high expression of HAS would cause an unbalance between HA precursors impairing HA-MM (Sheng et al., 2009). However Armstrong and Jonhs, (1997) concluded that in situations of glucose depletion this relation does not occur, according to Fig. (4a) the variation of the HA-MM is reduced in relation to μ_{max}.

With the increasing oxygen availability in the cultures, biosynthesis of biomass was benefited in relation to HA, with reduction of 86 % in $Y_{HA/Biomass}$ yield considering the results

of F1 and F4 cultures. As consequence of increased oxygen feeding in the cultures, there are three factors responsible for the reduction of the HA-MM and $Y_{HA/Biomass}$ yield; 1 –The effect of oxygen on the increase of acetate and ATP biosynthesis benefits biomass, consequently increasing HA production is observed for being associated to biomass (Jagannath and Ramachandran, 2010); 2 – Breaking of HA provided by ROS due to oxygen excess, as reported by Duan et al, (2009); 3 – Increased μ_{max} would cause the increasing concentration of HAS reducing HA-MM (Sheng et al., 2009).

The excess of oxygen also accelerated glucose consumption, being depleted before 20 h of cultivation in F1 to F4. In F4, the consumption of lactate and acetate was observed (Fig. 3b).

Fig.(5) shows the profiles obtained in medium composed by 70 g/L glucose and soy peptone at C/N ratio of 30.2 for F5 condition (D.O controlled at 90% saturation) and F6 condition with mixed D.O regime (uncontrolled up to 3 h and controlled at 90% saturation from 3 to 20h).

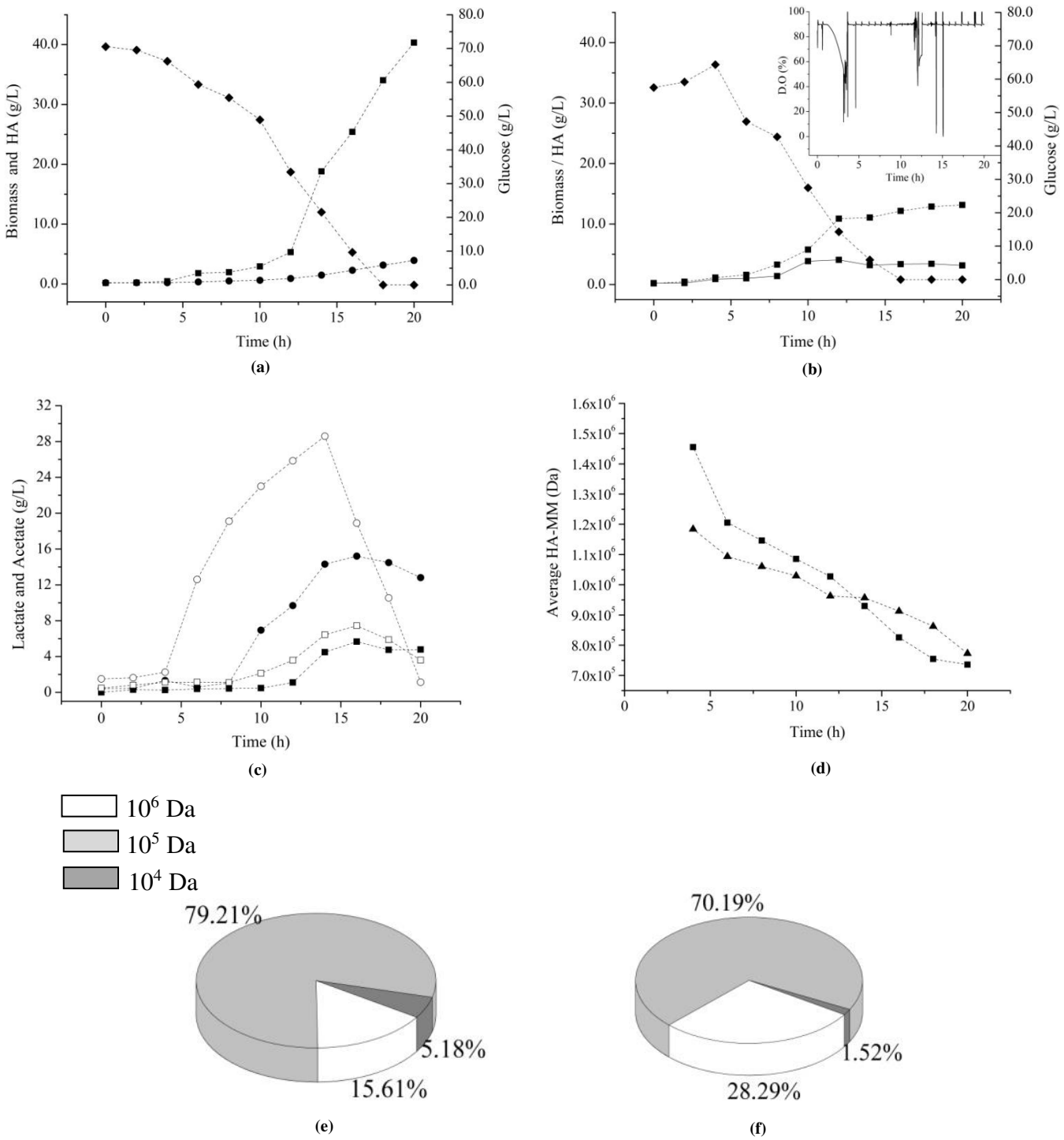

Fig. 5. Profiles of glucose (♦), biomass (■) and HA (●) at F5 condition (a) and F6 condition (D.O control inserted) (b), lactate (○, ●) and acetate (□, ■) at F5 (○, □) and F6 (●, ■) conditions (c), HA average molar mass at F5 (■) and F6 (▲) conditions (d), molar mass distribution at 20h for F5 condition (e) and molar mass distribution at 20h for F6 condition (f)

in cultivations of *Streptococcus zooepidemicus* ATCC 39920. F5 condition: dissolved oxygen control at 90% saturation. F6 condition: uncontrolled dissolved oxygen up to 3h and dissolved oxygen controlled at 90% saturation until 20h). For both cases culture medium was composed by 70g/L glucose and soy peptone at C/N ratio of 30.2 and controlled pH at 7.0.

The effects of the increased initial glucose concentration can be observed comparing the profiles at F5 (Fig. 5a) and F4 (Fig. 3a) conditions. In F5, glucose depletion (18h approx.) was prolonged, as expected, compared to F4 condition, due to its higher initial concentration (70 g/L). In addition, a longer lag phase (around 10 h) in F5 compared with F4 was observed, due to a probable catabolic inhibition at 70 g/L glucose, and effective glucose consumption around 25 g/L, as reported by Pires and Santana, (2010). With the increase of the initial concentration of glucose and maintaining the same conditions of D.O control in F5, the production of biomass (40.18 g/L) was 2.5 times higher than F4 (16.01 g/L). The cumulative HA concentration in 20h cultivation was 3.71 g/L in F5 which was 67% higher than F4 condition (2.35 g/L).

The metabolites lactate and acetate produced in F5 (Fig 5c) were not consumed in the initial phase of cultivation like in F4 (Fig 3b), due to the increment of glucose concentration. The average HA-MM was $7.3x10^5$ Da in F5 and $7.2x10^5$ Da in F4 condition. HA-MM distribution at the end of cultivation for F5 was 15.61% (10^6 Da), 79.21% (10^5 Da) and 5.18% (10^4 Da). Compared with F4 (Fig 3d), the increase of glucose concentration shifted the fractions to the greater HA-MM. Therefore, the increment of the initial glucose concentration from 25 g/L to 70 g/L with controlled D.O at 90% benefited biomass, HA production and HA-MM distribution. The average HA-MM could be benefited by ending fermentation around 10-12h.

The HA profile followed biomass behavior, reaching 3.71 g/L in F5 and 2.93 g/L in F6 condition. Besides biomass and HA production, the HA-MM distribution also

demonstrated a pronounced difference in F5 and F6 conditions (Fig 5e and 5f). The average HA-MM was $7.3x10^5$ Da in F5 and $7.7x10^5$ Da in F6 condition, however the HA-MM distribution in F6 shows an expressive increment in the 10^6 Da fraction (28.29%) compared to F5 (15.61%), at the expense of reducing of 10^4 Da fraction mainly, as 1.52% in F6 and 5.18% in F5. Therefore, the mixed D.O regime benefited not only the production of HA but also its quality, with fractions of higher HA MM.

Comparing the uncontrolled oxygen region in F6 (Fig. 5b), with the same situation in F3 (enriched air) (Fig. 1d), it could be observed higher initial D.O and glucose (90% and 70 g/L, respectively) in F6. As a consequence, biomass, HA and HA-MM were proportionally benefited, indicating the importance of the balance between glucose and oxygen in the cultivations.

In F6 condition, upon the oxygen excess provided by D.O control at 90% saturation, the biomass drastically changed their growth rate up to 20h (Fig. 5b) compared with F5 (Fig. 5a), suggesting a second lag phase, due to oxygen stress. According to Chong et al., (2005), HA production and biomass synthesis competes for limited nutrients in the culture medium, therefore reducing biomass synthesis with maintaining nutrient supply in the culture medium, more HA with higher HA-MM is produced. With the reduction of the synthesis of biomass in F6 $Y_{HA/Biomass}$ yield increased from 0.09 to 0.24 g/g (Table 3).

Table (3) shows the yields, μ_{max} and the average HA-MM results obtained from cultivations at F5 and F6 conditions. The data shows increased $Y_{HA/Biomass}$ yield from F6 > F4 > F5 conditions, with similar $Y_{HA/Glucose}$ yield for F5 and F6.

Table 3 – Yields, maximum specific growth rate (μ_{max}) and average HA molar mass (HA-MM) determined from cultivations of *S. zooepidemicus* ATCC 39920 with controlled dissolved oxygen at 90% saturation.

	F4	**F5**	**F6**
$\mathbf{Y_{HA/Biomass}}$ **(g/g)**	0.14	0.09	0.24
$\boldsymbol{R^2}$	0.915	0.983	0.773
$\mathbf{Y_{Biomass/Glucose}}$ **(g/g)**	0.62	0.51	0.21
$\boldsymbol{R^2}$	0.823	0.898	0.969
$\mathbf{Y_{HA/Glucose}}$ **(g/g)**	0.10	0.04	0.05
$\boldsymbol{R^2}$	0.916	0.889	0.778
$\boldsymbol{\mu_{max}}$ **(1/h)**	0.20	0.30	0.30
$\boldsymbol{R^2}$	0.846	0.971	0.968
Average HA-MM (x10^6)	0.72	0.73	0.77
SD	0.07	0.04	0.08

$Y_{Biomass/Glucose}$– Biomass produced per glucose consumption; $Y_{HA/Biomass}$– HA production per biomass produced; $Y_{HA/Glucose}$– HA production per glucose consumption; μ_{max} – maximum specific growth rate; F4– Glucose concentration at 25 g/L and dissolved oxygen control at 90 % saturation; F5 - Glucose concentration at 70 g/L and dissolved oxygen control at 90 % saturation; F6 - Glucose concentration at 70 g/L and dissolved oxygen control at 90 % saturation with mixed dissolved oxygen regime (uncontrolled up to 3h and afterwards controlled at 90% saturation).

According to the results obtained in this work, the presence of oxygen exhibited strong influence on biomass and HA production. The productivity in the absence of oxygen (F1) increased from 0.09 g/L.h to 0.12 g/L.h in the cultivation with D.O control at 90% (F4). Despite the same initial glucose concentration (25 g/L), the total glucose consumption occurred in 16 hours in F5, while in F4 the total consumption occurred in 8 hours of cultivation. With increasing glucose concentration in F5 (70 g/L) and D.O control at 90%, the

productivity increased to 0.18 g/L.h, exhibiting the importance of a balance between oxygen and glucose for increased productivity. Future studies involving the increasing microbial production of HA should focus on the optimization of the balance between glucose and D.O in the cultivations.

3.3. AMINO ACID PROFILE ANALYSIS

The analysis of amino acids were performed aiming to determine the amino acids involved in the increase of biomass (40.17 g/L) and HA (3.71 g/L) production observed in the F5 culture. Fig. (6) shows the concentration of 10 amino acids from soy peptone, which were considered essential for *Streptococcus zooepidemicus* ATCC 35246 growth according to Armstrong et al., (1997).

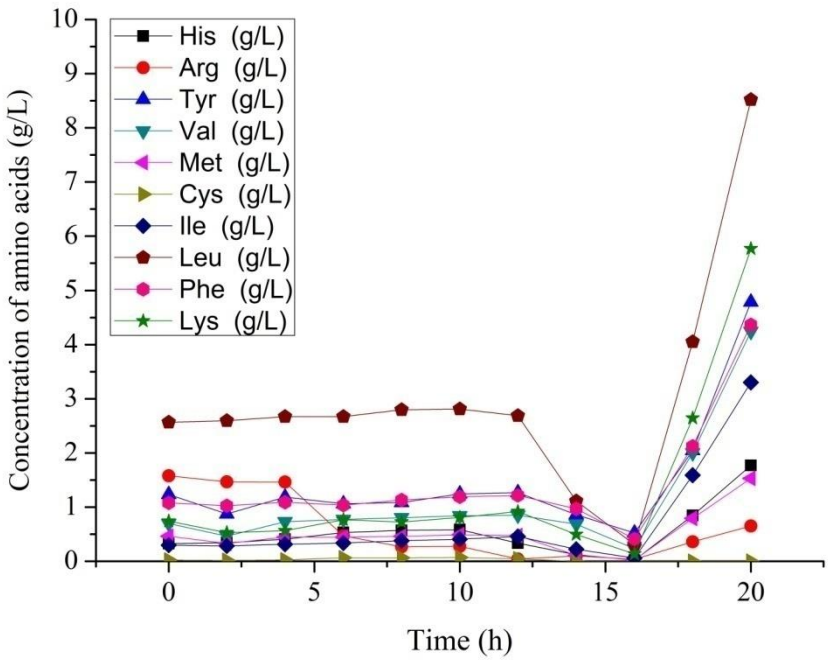

Fig. 6. Kinetic profiles of amino acids consumption during cultivation of *Streptococcus zooepidemicus* ATCC 39920, with culture medium composed of glucose (70 g/L) and soy peptone at C/N ratio of 30.2, with D.O controlled at 90%, control of pH at 7.0 and temperature at 37ºC during 20 hours of cultivation.

The profiles in Fig. (6) shows that the amino acids remained constant along the first 12 h (approx.), followed by a drop and further increasing in their concentrations. This behavior leads us to partially conclude that the production of proteolytic enzymes by the microorganism *Streptococcus zooepidemicus* ATCC 39920 is occurring. Kittang et al., (2017) reported that *S. zooepidemicus*is is capable of producing proteolytic enzymes.

From analysis of enzymatic activity in 3 points of F5 cultivation, it was observed the presence of proteolytic enzymes in the fermented broth: the results at the beginning of the cultivation was 0.37 U/mL, after 4 hours 1.08 U/mL and with 12 hours of cultivation this value rised to 2.38 U/mL. Therefore, the quantification of the amino acids consumed was impaired, since the effect of the enzymatic reaction could be occurring at different intensities along cultivation. The ability of the microorganism to produce proteolytic enzymes must be further studied in order to evaluate the efficiency of this mechanism in media with complex peptide chains.

These results are relevant because show the capability of *Streptococcus zooepidemicus* ATCC 39920 to modulate the uptake amino acids from complex nitrogen sources by means of the production of proteolytic enzymes.

4. CONCLUSIONS

The dissolved oxygen greatly influenced the glucose consumption, biomass and HA production in cultivations of *Streptococcus zooepidemicus* ATCC 39920. The balance between glucose and oxygen, as well as the oxygen control along cultivations modulated biomass and HA production. The mixed D.O control ensured the highest HA yield with less biomass production compared to the control along cultivation. In addition, the mixed regime favored HA-MM distribution, contributing to HA quality in terms of rheological properties. The biotechnological relevance of these findings is to understanding the modulation of HA production and its HA-MM in terms of glucose and D.O control for further optimization.

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Artigo 4

EFFECT OF PH ON ADSORPTION WITH ACTIVATED CARBON FOR PURIFICATION OF HYALURONIC ACID

Effect of pH on adsorption with activated carbon for purification of hyaluronic acid

R. C. Oliveira[1] • J. V. Moreira[1] • A. D. Cavalcanti[1] • M. H. A. Santana[1*]

[1]Department of Engineering of Materials and Bioprocesses, School of Chemical Engineering, University of Campinas, Albert Einstein Avenue, 500, Campinas - SP, 13083-852.

Abstract Hyaluronic acid (HA) is a biopolymer with several applications in the medical and cosmetic areas. Despite purification advantages in microbiological cultures, there are still challenges related to efficiency and cost reduction. In the present work the effect of pH on adsorption with activated carbon was studied as a complementary stage for HA purification. Precipitated solutions composed initially by HA (3.14 g/L) and total proteins and peptides TPP (1.56 g/L) were submitted to pH 6.0, 7.0 and 8.0 before adsorption with pulverized activated carbon at 0.25, 0.50, 1.00, 1.50 and 2.00% (w/v). Significant losses of HA were detected at pH 6.0 (around 62%), with activated carbon at 2.00%. Nevertheless at pH 8.0, the adsorption exhibited losses of HA close to 37%, increasing HA purity to 96% approximately, considering TPP as impurities only. Therefore adsorption with activated carbon can be used as a complementary stage of HA purification taking account the pH adjustment.

Keywords Hyaluronic acid – Purification - Vegetable peptones - Adsorption – Activated carbon

M. H. A. Santana*

1 - University of Campinas, School of Chemical Engineering, Department of Materials Engineering and Bioprocess, 500, Albert Einstein Avenue, 13083-852 - Campinas - SP - Brazil

Email: mariahelena.santana@gmail.com, Tel.: +55 19 3521 3921, fax: +55 19 3521 3890

1. Introduction

Hyaluronic acid (HA) is an endogenous glucosaminoglycan with many biological functions due to its unique hydrodynamic, viscoelasticity and molecular signaling properties. The HA is applied in medical, pharmaceutical and cosmetic areas, according to its purification grade and molar mass (HA-MM), leading a wide range of market values (US$ 2,000 to 60,000/kg) (Chong et al., 2005).

The microbial production of HA has become increasingly attractive for industries due to safe reasons, eliminating risks of viral contamination in animal tissues, such as rooster combs (Swan, 1968). The composition of the nitrogen source is an important parameter for cell growth and HA production; moreover, total peptides and proteins (TPP) also interfere in the final purity of the product (Guirard and Snell, 1962). Vegetable nitrogen sources are an alternative reducing nucleic acids and complex peptide chains in relation to the media composed by yeast extract or other animal sources (Ranish et al., 2003), thus the downstream process could be benefited with changes in the upstream related to the composition of the culture media.

Soy peptone has been used in microbial cultures because of its richness in essential free amino acids for microorganism growth and accomplishment of all metabolic functions. Liu et al. (2005) observed the metabolic effects with different nitrogen sources for nattokinase production by *Bacillus natto*. Using a Fractional Factorial Development (FFD), soy peptone and yeast extract was considered a promising culture medium, increasing nattokinase activity in 6 fold compared to the control medium. Fakhfakh-Zouari, (2010) studied the kerantinase production by *Bacillus pumillus* with different sources of nitrogen. The author concluded that the medium composed by soy peptone increases keratinase enzyme production in 3 fold if compared to the medium composed by yeast extract.

Despite the low TPP content in culture medium composed of vegetable sources, HA purification requires many steps to remove large amounts of impurities with high efficiency. Adsorption is used as an intermediate purification step, in which several adsorbents can be used, the activated carbon is a porous carbonaceous materials with a non-graphitic microcrystalline form, large porosity and surface area. Most carbonaceous materials have a surface area ranging from 10 to 15 m^2/g, and after activation with oxidation of the carbon atoms, activated carbon may have a surface area above 800 m^2/g (Boudou, 2003).

Rajendran et al., (2016) used *L. lactis* NZ9020 to produce HA in culture medium composed of 5 g/L brain heart infusion (BHI) and 5 g/L yeast extract. The cultivation broth was treated with 1% activated carbon for 2–3 h with continuous stirring, removing remaining protein and impurities. The concentration of proteins and nucleic acids in the solution obtained after adsorption treatment was almost negligible with HA losses ranging between 8 and 10 %. Choi et al, (2014) used *Streptococcus zooepidemicus* in cultivations with yeast extract (15 ~25 g/L) and glucose (60 ~ 80 g/L) in a 5 L bioreactor at 37°C and agitation rate of 300 rpm for 25 h. Activated carbon at 2.00% (w/v) was used to remove around 96% TPP and 90% endotoxins.

Considering this context, the HA purification by adsorption with activated carbon was studied in the present work. The production of HA was performed in 3.0 L bioreactor with *Streptococcus zooepidemicus* ATCC 39920 and culture medium composed by soy peptone and glucose at C/N ratio of 10.8. Dissolved oxygen was controlled at 90 % and pH at 7.0 during 20 hours cultivation. The adsorption process were carried out at pH 6.0, 7.0 and 8.0, observing the influence on HA purification with activated carbon at 0.25, 0.50, 1.00, 1.50, 2.00% (w/v).

2. Materials and methods

2.1. Microorganism

A strain of *Streptococcus zooepidemicus* ATCC 39920, belonging to the C group of Lancefeld was used on the production of HA. The native strain was obtained from the American Type Culture Collection (ATCC).

2.2. Inoculum and Fermentation

An ampoule containing the *Streptococcus equi* subsp, *zooepidemicus* ATCC 39920 culture was thawed and cultivated on Petri dishes containing soy peptone (67 g/L) and bacterial agar (30 g/L) (Benedini and Santana, 2013). After 24 hours of incubation at 37°C, submerged inoculum was prepared in Erlenmeyer flasks with culture medium composed of soy peptone and glucose (25 g/L), with a carbon/nitrogen ratio of 10.8 g/g. The first inoculum (25 mL) was kept under stirring at 150 rpm during 12 hours at 37°C. A second submerged inoculum stage (250 mL) was performed under the same conditions of stirring, temperature and culture medium, with a 10-fold increase in total volume of medium. After 6 hours of incubation, the second inoculum was transferred at 10% (v/v) to 3.0 L BIOFLO III bioreactor (New Brunswick ScientiCo, Inc, Edison, NJ, USA), with a work volume of 2.5 L. The temperature was maintained at 37ºC, with dissolved oxygen (D.O) controlled at 90% and pH at 7.0 during 20 hours of cultivation.

2.3. HA separation and purification

The fermented broth was centrifuged at 1,318 x g for 20 minutes. The cell-free supernatant was treated with 1.5/1.0 v/v ethanol (ethanol/supernatant). The solution was cooled at 4°C for 1 hour and then centrifuged for HA precipitation. The precipitate containing HA and total protein and peptide (TPP) was dissolved in 0.15 M NaCl. The precipitation process with ethanol and resuspension with NaCl was performed twice for HA and TPP quantification.

2.4. Adsorption isotherms

Powdered activated carbon Alpha LA 325 mesh (Alphacarbo industrial LTDA, Guarapuava, PR) was used in adsorption assays. The filtration of the activated carbon occurred through the use of qualitative filters CAT No. 1440-125 with 8 µm pore diameter (Whatman plc, Maidstone, UK).

After precipitation with ethanol, three Erlenmeyer flasks (100 mL) were separated with 50 mL of HA at 3.14 g/L and TPP at 1.56 g/L diluted in 0.15 M NaCl solution. The pH of the solutions was adjusted at 6.0, 7.0 and 8.0 with NaOH (0.1 M) and H_2SO_4 (0.1 M) solutions. Pulverized activated carbon at 0.25, 0.50, 1.00, 1.50, and 2.00 % (w/v) were used for adsorption isotherms curves. Activated carbon and prepared solutions containing HA were shaken at 150 rpm during 10 hours to reach equilibrium. For light scattering and HA-MM distribution analysis were used adsorption results with activated carbon at 2.00 % (w/v).

2.5. Analytical methods

2.5.1 Hyaluronic acid

The turbidimetric method was applied to determine the HA content according to Chen and Wang, (2009) studies. Standard of HA from Genzyme Corporation (Cambridge, MA, USA) was used in the method with readings at 400 nm.

2.5.2 Hyaluronic acid molar mass

The distribution of HA-MM was determined by size exclusion chromatography using a Shimadzu chromatographic system (Shimadzu Corporation, Kyoto, Japan) connected to a column guard (Polysep-GFC-P, 7.8 mm x 35 mm; Phenomenex, Torrance, CA, USA) and a gel filtration column (Polysep-GFC-P6000, 7.8 mm x 300 mm; Phenomenex, Torrance, CA, USA). The peak profile was monitored with a Shimadzu RID-6A refractive index detector (Shimadzu Corporation, Kyoto, Japan). Samples of 20 µL were injected, and $NaNO_3$ (0.1 M) was used as a mobile phase at 1.0 mL/min and 25°C.

2.5.3 Total protein and peptides in peptones

The TPP content in the solutions was determined by bicinchoninic acid assay (BCA; Pierce Chemical Company/Thermo Scientific, Rockford, IL). Nitrogen Kjeldahl used in the cultivation was calculated by dividing the TPP content in soy peptone by a factor of 6.25, as recommended by the Brazilian Health Regulatory Agency (Anvisa) ordinance No.360.

2.5.4 Light scattering and zeta potential

Electrophoretic mobility of the solutions was determined by Zetasizer 3000HS analyzer (Malvern, UK). Zeta potential and size distribution spectra were recorded.

3. Results and discussion

3.1. Adsorption isotherms

The pH effect on the adsorption of impurities composed by total proteins and peptides (TPP) was investigated after three consecutive steps of precipitation with ethanol in the ratio of 1.5/1.0 (ethanol/supernatant). The HA from the third precipitation was resuspended in 0.15M NaCl and used for construction of the adsorption isotherms with variation of pulverized activated carbon and pH. The concentrations of HA and TPP analyzed before isotherms assays were 3.14 g/L and 1.56 g/L respectively. Pulverized activated carbon was added at concentrations of 0.25, 0.50, 1.00, 1.50, 2.00 % (w/v) at pH 6.0, 7.0 and 8.0. The isotherms were carried out in steady state conditions.

Physical adsorption in activated carbon occurs through energy difference and forces of attraction (Van der Waals), that makes the molecules physically trapped in the activated carbon. The affinity of a substance by activated carbon is determined by its degree of ionization and water solubility. Poorly ionized hydrophobic substances are generally well adsorbed by activated carbon, in contrast hydrophilic substances that are ionized are not adsorbed (Hitchings et al., 2014).

Langmuir is the simplest and most widely used isotherm model in activated carbon isotherms, where each adsorption site is equivalent and independent (Langmuir, 1918). The Eq. (1) may present important information related to adsorption isotherms.

$$q_e = \frac{Q_{max} K_L C_e}{1 + K_L C_e} \quad (1)$$

C_e (g/L) is the equilibrium concentration of the sorbed components; q_e (g/g) is the adsorbed impurities concentration on the adsorbent surface at equilibrium; Q_{max} (g/g) is the amount of impurities adsorbed on the first layer of the adsorbent surface; K_L (L/g) is the adsorption constant and represents the degree of affinity of the adsorbate with the adsorbent.

The Fig. (1) shows the adsorption curves performed in 0.15M NaCl solution composed of HA and impurities (TPP) after previous purification with ethanol. The isotherms were constructed by the variation of activated carbon concentrations at pHs 6.0, 7.0 and 8.0.

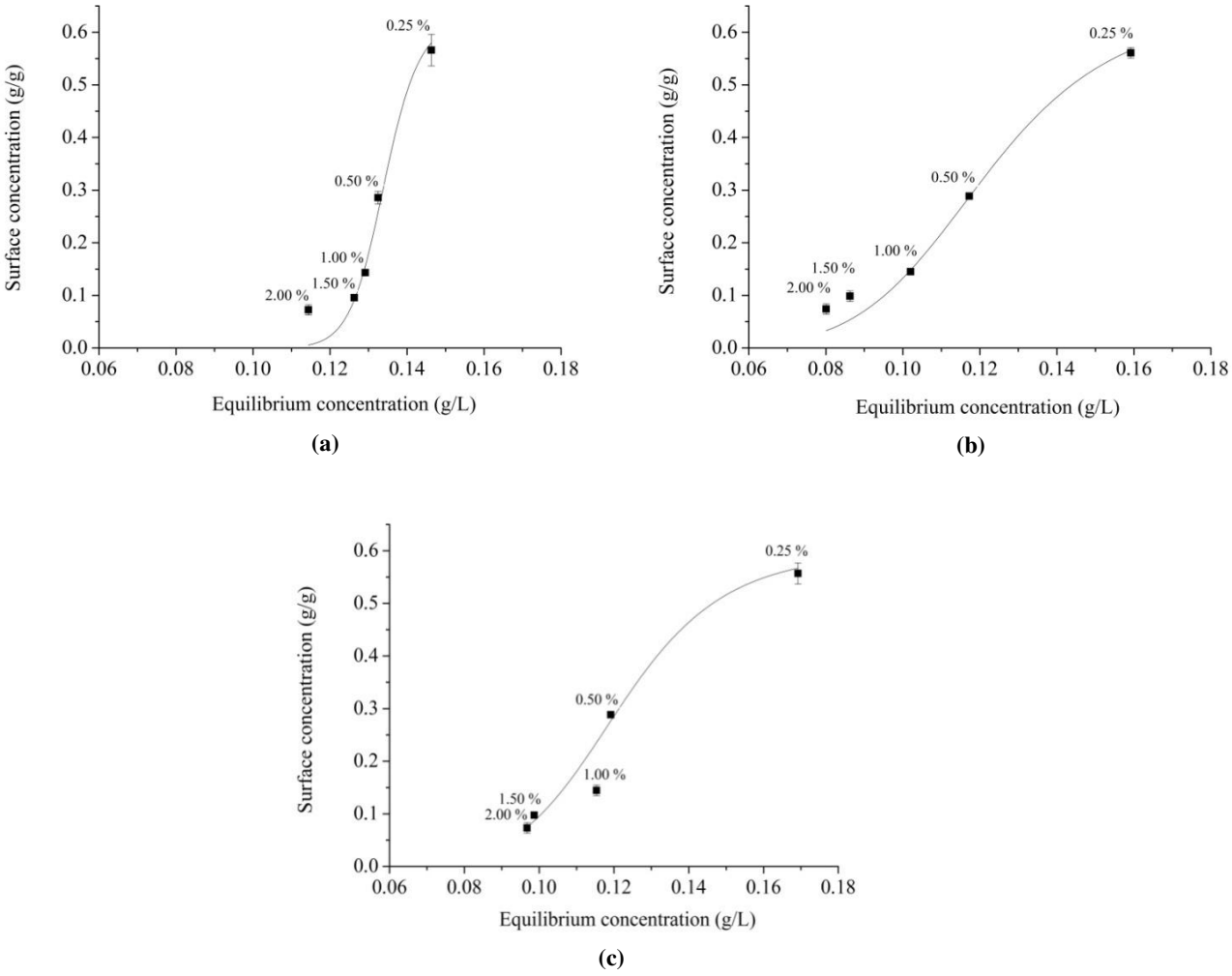

Fig. 1. Adsorption isotherms with 0.15M NaCl solution composed of HA (3.14 g/L) and total proteins and peptides (TPP) (1.56 g/L). The isotherms were constructed by the variation of activated carbon at 0.25, 0.50, 1.00, 1.50, 2.00 % (w/v) and pH at 6.0 (a), 7.0 (b) and 8.0 (c) at controlled temperature (25.0ºC). Surface concentration represents the amount in grams of TPP adsorbed per grams of activated carbon, Equilibrium concentration represents the concentration of TPP in equilibrium. Points: Experimental data; Lines: Langmuir adsorption isothermal model (OriginPro 8.5 Langmuir EXT1).

According to Fig. (1a), the isotherm at pH 6.0 exhibited greater slope, which may be related to the internalization of TPP into HA molecule, reducing the amount of TPP sorbed in the equilibrium as detected by the bicinchoninic acid method (BCA). The use of activated carbon at 2.0 % presented the best results in terms of purification, with reduced concentrations of TPP in the equilibrium solution.

Table (1) shows the parameters of Langmuir extracted from the adsorption isotherms performed at pH 6.0, 7.0 and 8.0 (Fig. 1).

Table 1 - Langmuir equation parameters extracted from performed isotherms at pH 6.0, 7.0 and 8.0 with variation of activated carbon at 0.25, 0.50, 1.00, 1.50, 2.00% (w/v).

pH	R^2	Q_{max} (g/g)	K_L (L/g)
6.0	0.755	0.624	$5.48x10^{25}$
7.0	0.983	0.646	$3.22x10^{6}$
8.0	0.849	0.599	$7.57x10^{7}$

The correlation of the adsorption at pH 6.0 was reduced compared to pH 7.0 and 8.0, with R^2 value of 0.755 (Table 1), indicating the interference of molecules of different sizes and charges presented by the soy peptone molecules. Newton et al., (2004) reported that vegetable cells may have more than 20000 different species of polypeptides, and therefore the interference due to the interaction of molecules of different sizes and charges is expected in the case of physical-chemical adsorptions. As shown in Table (1), the maximum adsorption values (Q_{max}) indicates similarities between values of 0.624, 0.646 and 0.599 g/g obtained in pH 6.0, 7.0 and 8.0 respectively, nevertheless K_L values indicates large favorable adsorption process at pH 6.0. The higher adsorption constant observed at pH 6.0 can be explained by the internalization of TPP caused by the attraction with HA molecules, increasing the adsorption of both components and also decreasing TPP as analyzed by the BCA method.

The BCA assay, first described by Smith et al, (1985), depends on the conversion of Cu^{2+} to Cu^{+}. By the biuret method, the ion Cu^{+2} reacts with the amine group of amino acids in protein chains, the resulted Cu^{+} is then detected by reaction with BCA. The high electronegativity of the HA prevents the transformation of Cu^{2+} into Cu^{+}, so the purple coloration in solution is not observed, the same could occur if the protein is in the internal region of the HA molecule.

The solution used in the adsorption contains macromolecules (HA, proteins and polysaccharides) as well as smaller molecules (free amino acids, dipeptides, tripeptides, etc). The zeta potential of soy peptone, HA and activated carbon were analyzed separately in Table 2 at pH 6.0, 7.0 and 8.0, in order to predict the behavior of the components when placed together.

Table 2 - Zeta potential analyzes performed in soy peptone, HA and activated carbon in solutions at pH 6.0, 7.0 and 8.0.

	Zeta Potential (mV)		
pH	**Soy peptone[a]**	**HA[b]**	**Activated carbon[c]**
6.0	-3.63	-18.15	-2.06
7.0	-6.07	-17.50	0.67
8.0	-7.27	-18.50	1.25

a: Soy peptone (1.0 g/l); b: HA (0.5 g/l); c: Activated carbon (1.0 g/l)

A considerable number of peptide chains present in soy peptone are composed of molecules with isoelectric point close to the studied pHs. Therefore a significant percentage of molecules may change their liquid charge according pH used in this work. Certain amino acids are determinants in the binding between HA and TPP, through ionic bonds with the carboxyl group of glucuronic acid (Kahmann et al., 2000). The binding between TSG-6 protein and HA is highly pH dependent. Changes in the histidine net charge showed maximum binding force in pH 6.0, and is reduced drastically with increasing pH (Parkar et al., 1998). Regarding to CD44 protein, arginine and tyrosine are of great importance in the binding with HA (Peach et al., 1993; Banerji et al., 1998). The number of CD44 residues indicates the existence of a specific interaction with the HA, in which the loss of a single hydrogen molecule or ionic interaction may be sufficient to reduce binding force (Bajorath et al., 1998).

3.2. Hyaluronic acid yield

With the liquid charge reduction in soy peptone solution at pH 6.0 (Table 2), the amount of molecules arranged to bind to HA is higher than pH 7.0 and 8.0. Although the formation of HA/TPP binding, amino acids chains in TPP molecules would remain exposed and therefore other bonds between HA and residual amino acids could occur (Gibs et al., 1968), altering the physical conformation of the HA molecule and the adsorption yield. Thus the yield of the adsorption process in relation to losses of HA must be taken into account.

The Fig. (2) shows the concentration of HA remaining after isotherms adsorption steps with activated carbon.

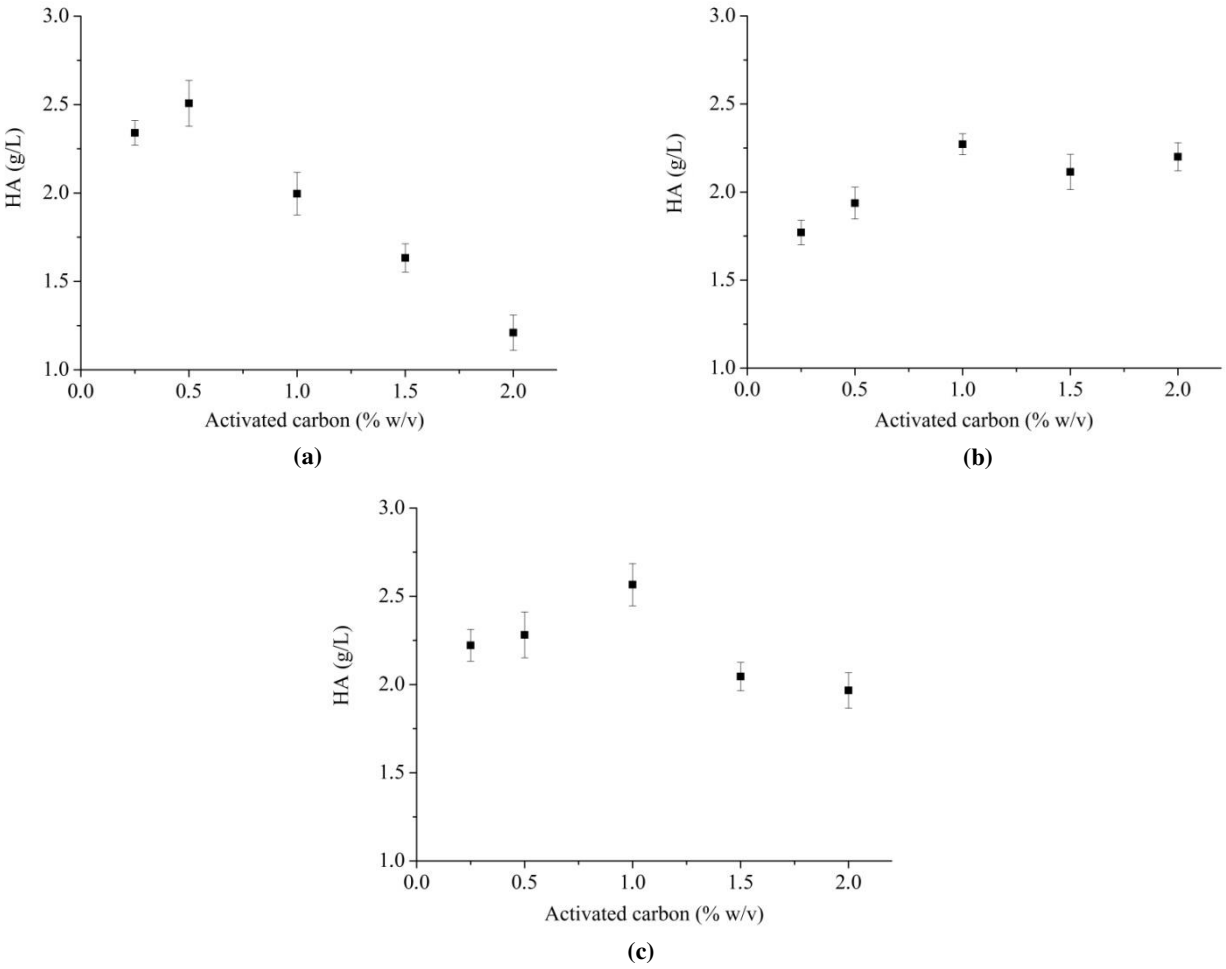

Fig. 2. Concentration of HA obtained in the sorbed solution after adsorption with pulverized activated carbon at 0.25, 0.50, 1.00, 1.50, 2.00% (w/v), pH at 6.0 (a); 7.0 (b) and 8.0 (c) and temperature controlled at 25.0°C. Initial 0.15 M NaCl solution composed by HA (3.14 g/L) and total proteins and peptides (1.56 g/L).

In the case of complex molecules such as proteins and HA, the charge and size of the molecules changes with pH, generating consequences for the adsorption efficiency. According to Fig. (2a), HA molecules were adsorbed along with TPP molecules. The internalization of TPP in HA molecules at pH 6.0 may have caused the adsorption of the HA/TPP complex reducing the efficiency of the HA recovery. However at pH 7.0 and 8.0, the electrostatic repulsion between TPP and HA molecules probably decreased the concentration

of adsorbed HA. Fig. (3) shows a model of HA/TPP complex in which the TPP internalization on HA decrease with increasing pH.

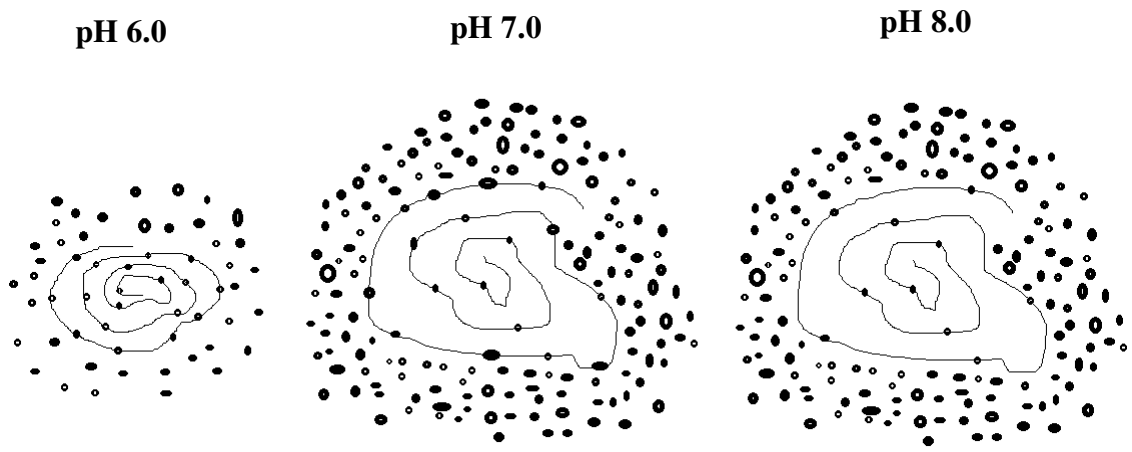

Fig. 3. Model demonstrating HA (—) and total protein and peptides (●) molecules behavior in solutions at pH 6.0, 7.0 and 8.0.

Some TPP molecules became positively charged at pH 6.0, then the attraction between TPP molecules and negatively charged HA could occur, with reduction in the size of the complex HA/TPP. Smaller particles would penetrate the pore of the activated carbon more easily, justifying the high loss of HA during adsorptions in pH 6.0. Al-Degs et al., (2008), observed that dye molecules with large hydrodynamic radius could not reach the micropores of the activated carbon resulting in an incomplete monolayer formation.

Balazs and Laurent, (1951) observed that ionized carboxyl groups of D-glucuronic acid are influenced by the ionic strength and pH of the environment, changing HA conformation and its interaction with neighboring molecules. In addition, internal electrostatic interactions between TPP residues and HA chain may contribute significantly to the reduction of the hydrodynamic radius of HA molecule. According to Gibbs et al., 1968, interactions between carboxyl group of HA with positive charges of some amino acids belonging to peptide chains bound to the HA chain can significantly alter the spatial morphology of the molecule.

Table (3) shows the losses and purity of HA at equilibrium after adsorption steps with activated carbon.

Table 3 - Losses and purity of HA in equilibrium after batch adsorption steps with activated carbon at 0.25, 0.50, 1.00, 1.50 and 2.00% (w/v).

	HA Losses (%)		
Activated Carbon %	pH 6.0	pH 7.0	pH 8.0
2.00	61.46 ± 2.65	29.97 ± 0.45	37.37 ± 1.33
1.50	48.01 ± 1.33	32.68 ± 0.68	34.87 ± 0.44
1.00	36.43 ± 0.88	27.67 ± 0.88	18.29 ± 2.65
0.50	20.16 ± 5.31	38.31 ± 0.26	27.36 ± 0.46
0.25	25.48 ± 0.44	43.63 ± 3.09	29.24 ± 0.44
	Purity (%)		
Activated Carbon %	pH 6.0	pH 7.0	pH 8.0
2.00	90.71 ± 2.14	95.80 ± 0.48	96.38 ± 0.50
1.50	92.42 ± 0.68	94.58 ± 2.12	95.95 ± 0.85
1.00	94.35 ± 0.10	96.18 ± 0.97	95.17 ± 1.74
0.50	93.29 ± 2.35	96.61 ± 0.45	95.31 ± 0.67
0.25	95.45 ± 0.29	93.31 ± 2.08	91.27 ± 3.07

Equations:
Purity (%) (Equilibrium HA / (Equilibrium HA + Equilibrium TPP))
HA Losses (%) (Initial HA (3.14 g/L) – Equilibrium HA) / Initial HA (3.14 g/L)

According to the results obtained at pH 6.0, the losses and purity of the HA in relation to TPP were 61.46% and 90.71% respectively, in adsorption with activated carbon at 2.00% (w/v). These results demonstrate the strong influence of pH on the recovery of HA, with increased HA losses after adsorption its purity relative to TPP would be impaired. The initial purity of HA, considering only TPP as a contaminant was 66.81%, demonstrates the activated carbon efficiency in the removal of TPP.

3.3. Size and HA molar mass

Fig. (4) shows the dynamic light scattering analyzes performed on the solution composed by standard HA and HA/TPP solution before and after adsorption steps with activated carbon at 2.00% (w/v) in pHs 6.0, 7.0 e 8.0. The concentration of activated carbon at 2.00% was used due to increased losses of HA at pH 6.0 (61.46%), in relation to the other pHs used in this work (Table 3).

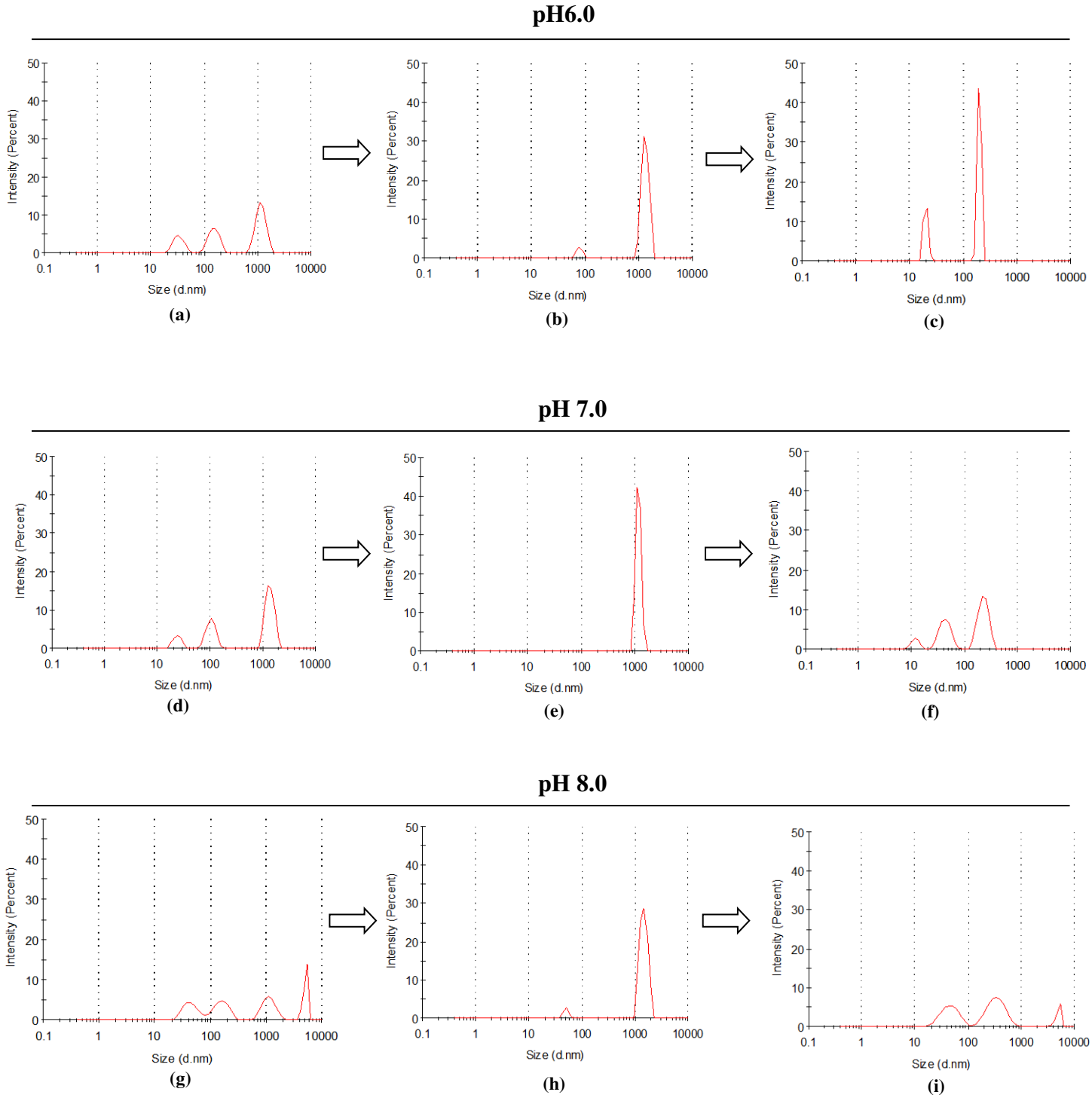

Fig. 4. Dynamic light scattering analyzes analysis in 0.15M NaCl solution composed of HA standard (0.5 g/L) in pH 6.0 (a), 7.0 (d) e 8.0 (g), precipited solutions composed of HA (3.14 g/L) and total proteins and peptides (1.56 g/L) in pH 6.0 (b), 7.0 (e) e 8.0 (h), and sorbed solution composed of HA and total proteins and peptides after the adsorption step with activated carbon at 2.00 % (w/v) in pH 6.0 (c), 7.0 (f), 8.0 (i).

With the presence of TPP at 1.56 g/L before adsorption with activated carbon, an increased hydrodynamic radius in relation to HA standard sample was observed in pH 6.0, 7.0 and 8.0, indicating complexation between TPP and HA molecules with hydrodynamic radius uniformity (Fig.s 4b, 4e and 4h). With the reduction of TPP content through adsorption on activated carbon, occurs the reduction of the hydrodynamic radius with the appearance of new populations and similar reading to those performed with the standard HA (Fig.s 4c, 4f and 4i). With the binding between TPP and HA molecules after adsorptions, the hydrodynamic radius of the HA molecule tends to decrease due to the cross-links between the TPP and HA chains as shown in Fig. (3). According to the results of number in light scattering analysis (data not shown), around 50% of the molecules present hydrodiamic radius of 3 nm at pH 6.0, while 23% of the molecules shows hydrodiamic radius of 23 nm at pH 8.0 followed by smaller percentages of populations, demonstrating increased dissociation and hydrodynamic radius of HA in pH 8.0, the same behavior was demonstrated by the Fig. (4g) with standard HA.

After adsorption at pH 6.0, one population of molecules with hydrodynamic radius of approximately 200 nm represent around 45% of the intensity (Fig. 4c), while at pH 8.0, the results showed the presence of three populations with intensity around 6%, proving the existence of free molecules with intermediates sizes (Fig. 4i). With increasing pH, the polydispersity of the hydrodynamic radius increases significantly, which may be related to the increased dissociation between TPP and HA molecules as shown in Fig. (3).

In addition to the changes in the hydrodynamic radius of HA molecules, its HA-MM should also be analyzed in relation to the effects of pH on the adsorption of TPP molecules. With the increasing attraction between opposite charges of TPP and HA molecules, long chains of HA could offer greater capacity of interaction with TPP, since they offer more active sites for HA/TPP binding (Hardingham and Muir, 1972).

Fig. (5) shows the distribution of HA-MM after adsorption on activated carbon at 2.00% (w/v) at pH 6.0, 7.0 and 8.0.

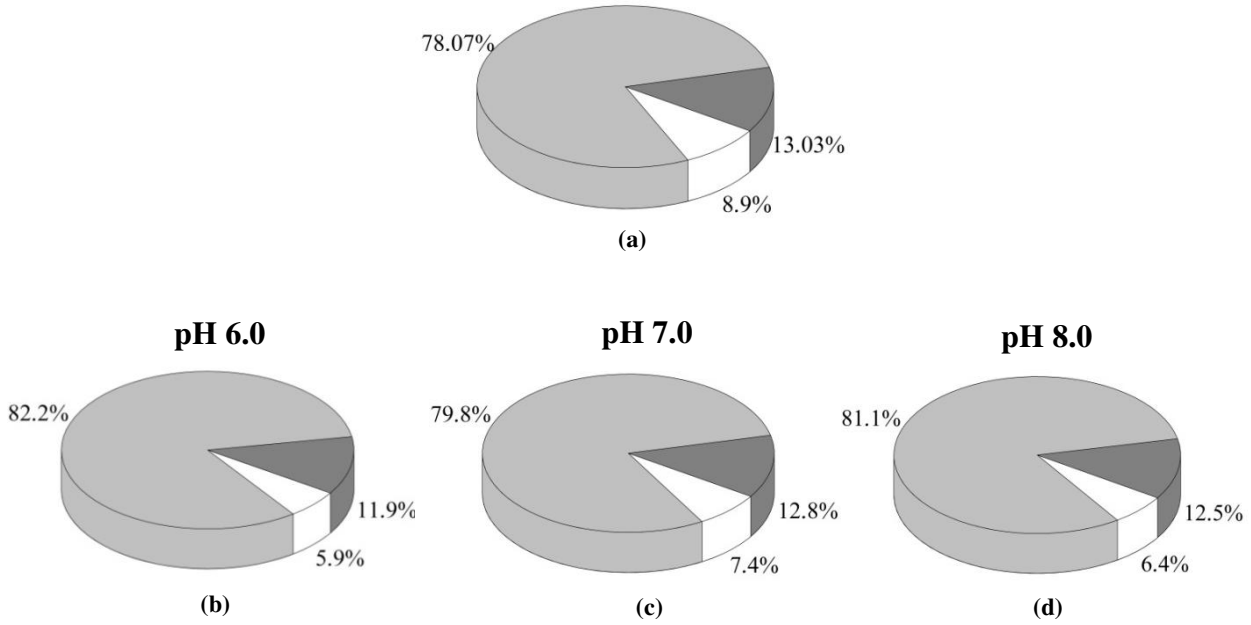

Fig. 5. Distribution of HA molar mass performed in the solution before (a) and after adsorption steps with activated carbon at 2.00 % w/v in pH 6.0 (b), 7.0 (c) and 8.0 (d). ☐ 10^6 Da; 10^5 Da; 10^4 Da.

According to Fig. (5), there were no significant differences between the HA-MM distribution. Similar average HA-MM were obtained before and after adsorptions assays at pH 6.0, 7.0 and 8.0 according to the Tuckey test at 95% confidence (data not shown).

The use of pulverized activated carbon is a valuable strategy for the purification of HA. The great difference of electronegativity and hydrodynamic radius between HA and TPP molecules facilitate the separation of impurities by adsorption. The conditions of pH must be controlled because they interfere in the recovery and yield of the process. At pH 6.0, the process generated losses of 61.4% in the initial concentration of HA, in assays at 2.00% w/v activated carbon. At pH 8.0 HA losses would reduce to 37.37%, with a reduction of 96.38%

in the initial concentration of TPP (Table 3). Adsorptions with activated carbon generated satisfactory conditions of separation between impurities (TPP) and HA between pH 7.0 and 8.0.

Future studies should be carried out in order to optimize the purification conditions of HA with activated carbon, taking into account the concentration of activated carbon , ionic strength and pH of the HA solution.

4. Conclusion

The pH control must be taken into account in adsorption with activated carbon for HA purification. The adsorption with activated carbon for HA purification has been shown an efficient process for the removal of total proteins and peptides with reduced changes for HA molar mass.

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Patente

PROCESSO PARA PRODUÇÃO E PURIFICAÇÃO DE ÁCIDO HIALURÔNICO POR VIA MICROBIANA

Processo para produção e purificação de ácido hialurônico por via microbiana

Número do Processo: BR 10 2017 027243 5

Data do depósito: 18/12/2017

Inventores:

RHELVIS DE CAMPOS OLIVEIRA[1] e MARIA HELENA ANDRADE SANTANA[1]

[1] Universidade Estadual de Campinas, Departamento de Engenharia Química

CAMPO DA INVENÇÃO - A presente invenção se insere no campo da biotecnologia, mais precisamente nas áreas médicas e farmacêuticas, e descreve um processo para a produção e purificação do ácido hialurônico por via microbiana (*Streptococcus zooepidemicus* ATCC 39920), com alto rendimento (acima de 2,5 g/L, podendo ser a partir de 1,5 g/L) e massa molar média alta (acima de 8,0 x 10^5 Daltons, podendo ser a partir de 5,0 x 10^5 Daltons), utilizando fontes de origem vegetal e de baixo custo a base de soja como única fonte de nitrogênio em todas as etapas do cultivo.

SUMÁRIO DE INVENÇÃO - A presente invenção tem por objetivo propor um processo de produção e purificação de ácido hialurônico a partir do cultivo da bactéria *Streptococcus zooepidemicus* ATCC 39920, utilizando a soja como única fonte de nitrogênio em todas as etapas do cultivo, reduzindo os custos de produção em relação ao comumente utilizado extrato de levedura e demais hidrolisados protéicos. Após o cultivo em batelada o ácido hialurônico produzido apresenta massa molar média maior que 8,0 x 10^5 Daltons e rendimento maior que 2,5 g/L. Adicionalmente, o número reduzido de etapas na purificação reduz os custos em relação às tecnologias existentes. A purificação contém apenas três etapas; centrifugação para a remoção da biomassa, precipitação do ácido hialurônico com etanol (1,5/1,0 etanol/sobrenadante) e adsorção de impurezas com carvão ativado. A concentração de proteínas e peptídeos totais após as etapas purificação apresenta valor inferior ou igual a 0,1%.

CONCLUSÕES

A razão entre aminoácidos livres e totais em peptonas de soja, assim como a suplementação do meio de cultura com cardiolipina e o controle do oxigênio dissolvido são estratégias para o aumento da massa molar do ácido hialurônico em cultivos com *Streptococcus zooepidemicus* ATCC 39920. A modulação da massa molar do ácido hialurônico é importante para atender aos requerimentos das aplicações médicas, farmacêuticas e em cosméticos.

A utilização do carvão ativado aliado ao controle de pH demonstrou resultados promissores em relação à remoção de proteínas e recuperação do AH, sem consequências para a sua massa molar.

Com relação aos aspectos específicos abordados neste trabalho concluímos que:

- A razão entre aminoácidos livres e totais determina a absorção do nitrogênio de peptonas de soja com maior influência sobre a taxa específica de crescimento celular e a massa molar do ácido hialurônico. Em meios de cultura com déficit de aminoácidos livres, o aumento da sua fração em relação aos aminoácidos totais reduziria a taxa específica de crescimento celular com aumento da massa molar do ácido hialurônico.
- Os dados experimentais demonstram aumento na concentração e massa molar do AH com a adição de cardiolipina a 30 mg/L, em meio de cultura composto por peptona de soja e glicose ou extrato de levedura e glicose.
- O balanço entre os fornecimentos de glicose e oxigênio ao longo dos cultivos demonstra ter importância fundamental no controle do fluxo metabólico para a produção de biomassa e AH. O controle misto de O.D resultou no aumento da massa molar do AH com redução da biomassa produzida.
- A produção de enzimas proteolíticas pelo microrganismo *Streptococcus zooepidemicus* ATCC 39920 tornaria desnecessária a utilização de hidrolisados enzimáticos como fontes de nitrogênio, reduzindo substancialmente os custos com meio de cultura para produção microbiana do AH.
- O controle de pH aliado à adsorção com carvão ativado para a purificação do AH se mostrou eficiente na remoção de proteínas e peptídeos totais e recuperação do AH produzido.

SUGESTÕES PARA TRABALHOS FUTUROS

- Estudos realizados com fontes complexas de nitrogênio devem levar em consideração a presença de açúcares em sua composição, analisando o seu consumo durante o cultivo e avaliando a sua influência na produção do AH.

- A razão cardiolipina/biomassa deve ser otimizada no sentido de elevar a concentração e massa molar do AH produzido com cultivos em biorreator.

- Estudos objetivando o aumento da produção de AH e do rendimento em relação à biomassa devem se concentrar na otimização do balanço glicose/oxigênio nos cultivos.

- A quantificação dos aminoácidos consumidos deve ser realizada a partir de cultivos com meio de cultura definido, com o objetivo de identificar os aminoácidos responsáveis pela variação da produção e massa molar do AH.

- Estudos futuros devem ser realizados com o objetivo de otimizar a eficiência da purificação do AH em carvão ativado através de modificações no pH e força iônica.

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