UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
DOUTORADO EM CIÊNCIAS BIOLÓGICAS

PRODUÇÃO E PURIFICAÇÃO DE DNA PLASMIDIAL A PARTIR DE *Escherichia coli* RECOMBINANTE

KEILA APARECIDA MOREIRA

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Tese apresentada ao Curso de Doutorado em Ciências Biológicas da Universidade Federal de Pernambuco, para obtenção do título de Doutor em Ciências Biológicas, Área de Concentração em Biotecnologia.

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(Departamento de Morfologia e Fisiologia Animal – UFRPE)

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DNA – ácido desoxirribonucléico

RNA – ácido ribonucléico

ETDA – ácido etilenodiaminotetracético

NaOH – hidróxido de sódio

SDS – dodecil sulfato de sódio

PEG – polietileno glicol

NaCl – cloreto de sódio

$MgCl_2$ – cloreto de magnésio

CFR – cromatografia de fase reversa

CIH – cromatografia de interação hidrofóbica

SDFA – sistemas de duas fases aquosas

K- coeficiente de partição

RESUMO

A produção e a purificação de DNA plasmidial com elevado grau de pureza tem tido um elevado interesse nos últimos anos, devido fundamentalmente aos desenvolvimentos de técnicas de medicina molecular, como a terapia gênica e a vacinação com DNA. O plasmídeo pD2 foi produzido em diferentes condições de cultivo, sendo analisado várias parâmetros durante o crescimento, tais como, influência da velocidade de agitação (120, 160 e 200 rpm), concentrações de canamicina (10, 20, 30, 40 e 50μg ml^{-1}) e meio de cultura (Terrific Broth - TB ou Luria Bertani com glicose - LBG). Foram também investigados o efeito da partição de DNA plasmidial, RNA e proteínas totais nos sistemas de duas fases aquosas (SDFA) do tipo polietileno glicol (PEG)/sal de fosfato de sódio dibásico (K_2HPO_4) do plasmídeo (pD2) durante o processo de purificação. Analisou-se a influência da massa molar do PEG, massa que carrega o sistema da solução de lise (20, 40 ou 60% m/m) na partição do plasmídeo. A técnica de precipitação com sulfato de amônio (2,0, 2,5 e 3,0M), seguido da técnica cromatográfica de interação hidrofóbica (CIH) utilizando como matriz estacionária o suporte Phenyl Sepharose 6 Fast Flow foram utilizadas para purificar o plasmídeo pVax-LacZ. A velocidade de agitação que apresentou os melhores resultados para a produção de biomassa e DNA plasmidial foi a 200 rpm, enquanto as concentrações 30-50μg mL^{-1} de canamicina apresentaram resultados semelhantes para todas as condições estudadas. O meio de cultura TB apresentou ser o melhor em termos de produção de DNA plasmidial e biomassa, neste meio de cultura o plasmídeo foi mais estável, apresentando 82% de células contendo o plasmídeo. O DNA plasmidial foi particionado entre a fase superior e inferior na dependência da massa molar do polímero constituinte do sistema, tendo uma grande quantidade de proteína do lisado celular acumulada na interfase dos sistemas. O melhor rendimento plasmidial (37%) foi obtido com sistema PEG 400/K_2HPO_4 (20/20% m/m) carregado com 60% (m/m) de lisado celular. O procedimento de precipitação com sulfato de amônio (2,5M) seguido da cromatografia de interação hidrofóbica foram capazes de separar proteínas e DNA genômico do plasmídeo, obtendo 51% de rendimento e um fator de purificação de 3,3.

ABSTRACT

The production and purification of plasmid DNA with high degree of purity have been having a large interest in the last years, mainly due to the development of several techniques, such as molecular medicine, gene therapy and DNA vaccination. The different bacterial growth conditions were set up in order to analyze the plasmid pD2 production, such as the influence of agitation velocities (120, 160 and 200 rpm), kanamycin concentrations (10, 20, 30, 40 and 50μg ml^{-1}) and culture medium (Terrific Broth - TB or Luria Bertani with glucose - LBG). Were also investigated the effect of plasmid DNA, RNA and total proteins in the aqueous two phases systems (ATPS), polyethylene glycol (PEG)/ sodium phosphate salt (K_2HPO_4) on the plasmid (pD2). Was also analyzed the influence of the molecular weight of PEG, load of lyse solution (20, 40 or 60% w/w total mass of the systems) on the plasmid DNA partition. With the pVax-LacZ plasmid the precipitation with ammonium sulphate (2.0, 2.5 and 3.0M) followed by hydrophobic interaction chromatography (HIC) using as support Phenyl Sepharose 6 Fast Flow was applied. The agitation velocity that presented better results related to biomass and plasmid DNA production was 200 rpm, while 30-50μg mL^{-1} of kanamycin presented similar results for all the studied conditions. The TB medium was the best medium for biomass and DNA production. The plasmid was stable in TB medium, showing 82% plasmid containing cells. The plasmid DNA are partitioned between the top an bottom phases showing to be dependent to the molecular weight of the system and a good amount of cell lysate protein was accumulated in the interphase of the system. The best recovery plasmid yield (37%) was obtained with PEG 400 system with a 60% (w/w) lysate load. The precipitation procedure with ammonium sulphate (2.5M) followed by the hydrophobic interaction chromatography were capable to separate proteins and genomic DNA of the plasmid, obtaining 51% of revenue and a purification factor of 3.3 after the precipitation with ammonium sulphate.

1. INTRODUÇÃO

Plasmídeos são elementos genéticos constituídos por moléculas de DNA extracromossômicas em cadeia dupla covalentemente fechada que podem existir em diferentes conformações topológicas e são capazes de manter-se estáveis em populações microbianas (SINDEN, 1994; GANUSOV; BRILKOV, 2002).

Todos os plasmídeos possuem uma seqüência de DNA que pode atuar como origem de replicação, de modo que eles são capazes de se multiplicar na célula independentemente da molécula principal de DNA. Os plasmídeos menores fazem uso das próprias enzimas replicativas de DNA da célula para fazer cópias de si mesmos, ao passo que alguns dos plasmídeos maiores possuem genes que codificam enzimas especiais, específicas para sua replicação (BROWN, 1999).

Alguns plasmídeos também são capazes de se replicar inserindo-se na molécula principal de DNA. Esses plasmídeos são integrativos ou episssomos, podem ser mantidos de modo estável durante várias multiplicações celulares, mas sempre em algum estágio existem como elementos independentes (PROCTOR, 1994; BROWN, 1999).

Os plasmídeos podem ser categorizados em dois grandes grupos, os plasmídeos conjugativos ou não conjugativos, dependendo se transportam ou não um conjunto de elementos utilizados na transferência de genes que promovem a conjugação bacteriana (Tabela 1). Estes ainda podem ser agrupados em relação à quantidade de cópias que eles podem manter por células, múltiplas cópias ou limitadas cópias (OLD; PRIMROSE, 1981).

Geralmente os plasmídeos conjugativos são de alta massa molar e estão presentes com uma a três cópias por cromossomo, enquanto que os plasmídeos não conjugativos são de baixa massa molar e estão presentes com múltiplas cópias. Eles ainda podem ser classificados de acordo com o controle preferencial na duplicação, plasmídeos

restritos ou relaxados. Os primeiros duplicam-se em sincronia com o cromossomo, enquanto que os plasmídeos relaxados duplicam-se independentemente da coordenação cromossômica (OLD; PRIMROSE, 1981).

A partir de 1946, quando Lederberg e colaboradores descreveram o plasmídeo F, responsável pela fertilidade em *Escherichia coli* K12, centenas de outros plasmídeos foram descritos, alguns deles codificando informações de grande relevância para a saúde humana, animal e vegetal, como os plasmídeos R (resistência múltipla a drogas) em diversos grupos bacterianos Gram-negativos e positivos. Mais de 250 plasmídeos já foram descritos somente em *Escherichia coli.* Além desses plasmídeos naturais, existem dezenas de plasmídeos artificiais (COSTA, 1987).

Tabela 1. Propriedades de plasmídeos conjugativos e não-conjugativos de organismos Gram-positivos.

Plasmídeo	Tamanho (Kb)	Conjugativo	Número de cópias plasmídeos/cromossomo	Fenótipo
Col E1	4,2	Não	10-15	Produção de colicina E1
RSF 1030	5,6	Não	20-40	Resistência a ampicilina
Clo DF13	6	Não	10	Produção de cloacina
R6K	25	Sim	13-28	Resistência a estreptomicina e ampicilina
F	62	Sim	1-2	----------
RI	62,5	Sim	3-6	Resistência múltipla a drogas
Ent P 307	65	Sim	1-3	Produção de enterotoxina

Fonte: OLD; PRIMROSE, 1981.

Os plasmídeos são comumente conhecidos como veículos para introdução de novos genes dentro de uma célula viva (BAHERI et al., 2001). Estes são freqüentemente usados como modelo para estudos de replicação, recombinação e segregação de DNA em células microbianas. O DNA plasmidial tem adquirido recentemente considerável interesse devido ao seu potencial de aplicação em terapia gênica e vacinas de DNA (PRAZERES et al., 1999; LEVY et al., 2000; FERREIRA et al., 2000; WANG et al., 2001; RIBEIRO et al., 2002).

1.1 Caracterização estrutural dos plasmídeos

As moléculas de DNA são hélices formadas por duas cadeias alinhadas segundo um eixo de forma antiparalela. Cada cadeia é um polímero linear de nucleotídeos ligados entre si por ligações fosfodiéster. Na estrutura da molécula de DNA as bases nitrogenadas encontram-se empacotadas junto ao centro do eixo da hélice, enquanto que no exterior da hélice se encontram os grupos fosfato, negativamente carregados. O interior da hélice é, portanto, altamente hidrofóbico devido à presença das bases aromáticas (SINDEN, 1994).

A estrutura em cadeia dupla é altamente estável. Esta estabilidade é dada através das forças de empacotamento (hidrofóbicas e de van der Walls) entre as bases da molécula, mas também das ligações de hidrogênio e da camada de moléculas de água que solvatam o DNA. A desnaturação do DNA (separação das cadeias) pode resultar da quebra das ligações de hidrogênio a pH<2 ou pH>12 devido à ionização das bases, do aumento da temperatura ou da força iônica e, ainda, de cortes ou rearranjos na sua estrutura (RIBEIRO, 2000).

Na molécula do plasmídeo, se o eixo da hélice do DNA estiver também enrolado, forma-se uma estrutura altamente ordenada, superenrolada (Figura 1); se o plasmídeo sofrer um corte numa das cadeias, a estrutura superenrolada perde-se, resultando numa

estrutura circular aberta; se o corte for em ambas às cadeias, à estrutura passa a ser linear. A forma circular aberta inclui também moléculas que não têm cortes nas suas cadeias e às quais se dá o nome de relaxadas. As moléculas de plasmídeo extraídas de uma bactéria encontram-se naturalmente na forma superenrolada, mas existe também uma fração na forma aberta (SINDEN, 1994).

A estrutura que corresponde a maior estabilidade para o plasmídeo, ou seja, a menor energia, é a forma circular aberta. No momento, *in vivo,* injúrias à molécula impõem a forma superenrolada. Como as moléculas de plasmídeo são polianiônicas a força iônica do ambiente que o rodeia tem um efeito profundo na sua estrutura (McFAIL-ISOM et al., 1999).

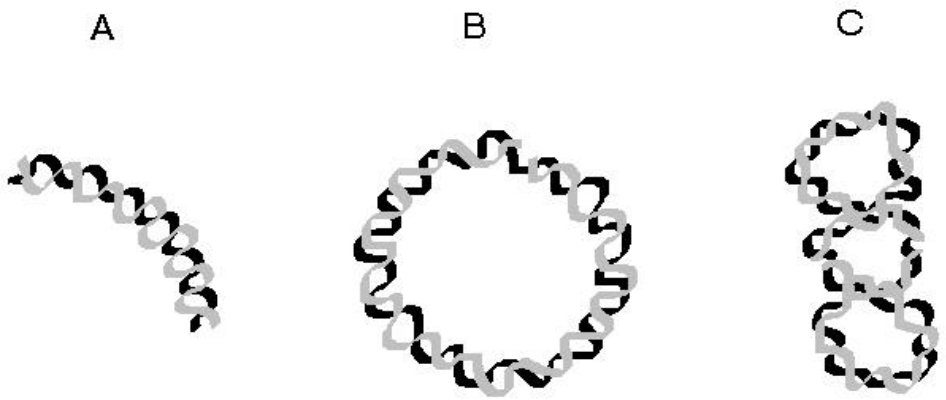

Figura 1. Exemplo de diferentes conformações do plasmídeo: A) linear; B) circular aberta; C) superenrolada.

Em elevadas concentrações de sal, o plasmídeo está altamente compactado, com as bases hidrofóbicas empacotadas e blindadas no interior da hélice. Isto se deve ao efeito dos cátions presentes em solução, que reduzem a repulsão eletrostática entre os grupos fosfato do DNA (SINDEN, 1994). Se a quantidade de sal for reduzida, as repulsões eletrostáticas dominam e a molécula torna-se menos superenrolada e mais aberta (SHLICK; OLSON, 1994).

1.2 Produção de DNA plasmidial

O sucesso de fermentações baseadas em tecnologias de DNA recombinante é uma combinação das interações entre o ambiente fermentativo, o organismo hospedeiro e os elementos genéticos recombinantes (O' KENNEDY et al., 2000). A produção de plasmídeos por processos fermentativos em larga escala podem apresentar limitações quanto ao baixo rendimento, instabilidade plasmidial e altas densidades celulares (DIOGO, 1999).

A produção de plasmídeos inclui três fases principais: a fermentação, o isolamento primário e a purificação. Em cada uma destas etapas existem problemas específicos, na sua maioria relacionados com a natureza estrutural dos plasmídeos, como a elevada massa molar (normalmente entre 5 a 20 kilobases), o que resulta em soluções muito viscosas e uma estrutura não globular flexível e altamente carregada (PRAZERES et al., 1999).

Meios de cultura comerciais também podem ser utilizados, sendo recomendado o desenvolvimento de meios que sejam adaptados ao sistema plasmídeo/hospedeiro em questão, de modo a aumentar a produtividade (PRAZERES et al., 1998).

Deve-se ter cuidados especiais com a escolha de uma espécie como hospedeira de um plasmídeo, de maneira a obter rendimentos e prevenir alguns problemas em etapas posteriores, como a purificação. Assim, a espécie deve ser escolhida para minimizar a quantidade de impurezas que necessitam ser removidas (PRAZERES et al., 1999). Em geral, deve ser escolhida uma espécie hospedeira que tenha sido completamente caracterizada, que esteja livre de qualquer contaminação e que não seja prejudicial para o ambiente, para o produto final, para os pacientes e para pessoal envolvido na produção e manipulação (SCHLEEF, 1999).

Trabalhos anteriores envolvendo tecnologias de DNA recombinante focalizam seus estudos em seres procariontes, como a bactéria *Escherichia coli*. Esta é

considerada como um hospedeiro ideal, pois tem reconhecida segurança ao longo da história científica na utilização em produtos farmacêuticos (VYAS et al., 1994; ZHANG et al., 1996).

1.3 Estabilidade plasmidial

Na produção comercial com microrganismos recombinantes, a maioria dos problemas está na estabilidade plasmidial. A instabilidade é a tendência das células transformadas perderem suas propriedades de engenharia molecular por causa de mudanças ou perdas do plasmídeo (ZHANG et al., 1996). A instabilidade plasmidial em cultura microbiológica provoca redução dos níveis do produto desejado no cultivo, tendo impacto negativo em atividades de proteínas específicas e aumento nos custos de produção, visto que os substratos são consumidos por células não produtivas (VYAS et al., 1994).

Existem dois tipos de instabilidade plasmidial: a estrutural e a segregacional. A instabilidade estrutural é causada usualmente pela deleção, inserção, recombinação ou outros eventos, ao nível de DNA; enquanto que a instabilidade segregacional é causada pela partição desigual de plasmídeos durante a divisão celular (OLD; PRIMROSE, 1981).

Após o plasmídeo recombinante ser introduzido dentro de células hospedeiras, as interações entre plasmídeo e o hospedeiro são substanciais (Tabela 2). Estas interações determinam a instabilidade plasmidial e o grau de expressão dos genes clonados. Portanto, estes fatores combinados a fatores ambientais podem levar a perda plasmidial (MÔO-YOUNG et al., 1996), resultando em perda da produtividade do produto desejado, sendo um grande obstáculo para o aumento da escala no uso de microrganismos geneticamente modificados (ZHANG et al., 1996).

Tabela 2. Fatores que afetam a estabilidade plasmidial em leveduras.

Fatores genéticos	Arranjo do plasmídeo
	Número de cópias do plasmídeo
	Nível de expressão
	Marca seletiva
	Propriedades das células hospedeiras
Fatores ambientais	Formulação do meio de cultura
	Tensão do oxigênio dissolvido
	Temperatura
	Taxa de diluição
	Modos de operação do biorreator

Fonte: ZHANG et al. 1996

1.4 Marca seletiva

Uma marca seletiva é necessária para identificar os clones transformados. A presença de um marca seletiva permite a manutenção estável se uma pressão de seleção for imposta. Genes que conferem resistência a antibióticos são marcadores comumente usados. Tais genes são inseridos nos plasmídeos e os vetores resultantes são usados para transformação. Meios de cultura seletivos contendo, quase na sua obrigatoriedade, antibióticos são usados para eliminar células livres de plasmídeos enquanto mantém as células transformadas. Antibióticos normalmente são caros, e a sua presença pode prejudicar a recuperação do produto final (JIMINEZ; DAVIES, 1980).

2. PROCESSOS DE PURIFICAÇÃO

Os maiores obstáculos à produção de plasmídeos em larga escala encontram-se associados aos processos de purificação, embora a tecnologia de fermentação utilizada ainda necessite de vários aperfeiçoamentos (CARLSON et al., 1995). Os custos de

produção de proteínas terapêuticas, cerca de 70%, podem estar associados às operações de separação e purificação. Parece ser improvável que as especificações de pureza e custos de recuperação venham a ser menor no caso dos vetores de terapia gênica (LYDDIATT; O'SULLIVAN, 1998).

2.1 Isolamento primário

As dificuldades mais comuns encontradas no isolamento primário de plasmídeos são: a alta densidade celular, tensões de corte elevada, desnaturação, ação das nucleases, manipulação dos lisados e fragmentação do DNA genômico (PRAZERES et al., 1999).

A etapa de recuperação das células ao final da fase de produção de microrganismos recombinantes, inicia-se por operações de separação sólido-líquido, ocorrendo através de centrifugação ou microfiltração. A ressuspensão dessas células é feita em tampão que contém ácido etilenodiaminotetracético (EDTA), usado como agente quelante. O EDTA remove íons de Ca^{2+} e Mg^{2+} da superfície celular, desorganizando a sua estrutura e reduzindo a atividade das nucleases que podem degradar o plasmídeo (PRAZERES et al., 1999).

A técnica de lise alcalina inicia-se pela adição e agitação suave de um determinado volume de células com uma solução de hidróxido de sódio (NaOH) contendo dodecil sulfato de sódio (SDS). A reação de lise na parede celular, entre o SDS e os lipídios e as proteínas, solubiliza o material da parede celular, provocando liberação do conteúdo intracelular. Resultados de alguns pesquisadores parecem indicar que a lise química da parede celular e a liberação dos materiais intracelulares se completam ao fim de 30-40 segundos dependendo da espécie bacteriana (CICCOLINI et al., 1998).

O ambiente fortemente alcalino da mistura causa também a desnaturação reversível do DNA plasmidial. O DNA cromossômico de alta massa molar é também desnaturado nesta altura causando o aumento da viscosidade da solução até um máximo,

antes de diminuir para um estado estacionário. Esta diminuição é resultante da fragmentação do DNA genômico induzida pela tensão de corte (CICCOLINI et al., 1998).

Quando a lise e a desnaturação ficam completas, a mistura é neutralizada pela adição de uma solução concentrada de acetato de potássio. De fato, a solubilização do SDS decresce a baixas temperaturas e as altas concentrações de sal, resultando na formação de uma suspensão floculenta, formada por complexos SDS-proteínas. Estes flocos, muito sensíveis às tensões de corte, vão se agregando lentamente, formando uma matriz contendo debris celulares, RNA, DNA genômico de alta massa molar e outras impurezas que vão ficando retidas nesse reticulado. Os plasmídeos, majoritariamente dissolvidos na fase aquosa, recuperam a forma original superenrolada nesta fase do processo o que lhe permite resistir melhor às tensões mecânicas. O precipitado formado é separado da fase aquosa por centrifugação ou filtração. Nesta operação pode ser perdido algum DNA plasmidial, o que pode ser minimizado removendo o máximo de líquido da fase sólida (PRAZERES et al., 1999).

Ao longo de todo o processo de purificação, o processo de lise alcalina é um dos passos de maior dificuldade. A literatura descreve métodos alternativos para isolamento primário dos plasmídeos, nomeadamente a sonicação e a homogeneização a alta pressão (CARLSON et al., 1995). Todos os métodos testados provocaram uma grande fragmentação do DNA genômico, o que constitui um problema adicional em termos dos passos subseqüentes (THEODOSSIOU et al., 1997).

A liberação de DNA genômico fragmentado aumenta a viscosidade da solução, tornando o processo de mistura mais difícil e dispendioso. Adicionalmente, a agitação deve ser feita de forma suave, de modo a manter o DNA genômico com a massa molar o mais alto possível de modo a maximizar a sua precipitação nas etapas seguintes

(RIBEIRO, 2000).

Em contraste, a adição dos reagentes que provocam a lise celular deve ser suficientemente eficaz de modo a evitar a formação de locais com extremos valores de pH, uma vez que valores superiores a 12 poderão provocar a desnaturação irreversível dos plasmídeos, passando estas isoformas a ser consideradas contaminantes do processo. A agitação deve também assegurar a lise completa e eficiente de toda a população celular. A lise alcalina é um processo difícil de controlar, apresenta falta de reprodutividade e pode implicar perdas significativas de plasmídeo (PRAZERES et al., 1999).

2.2 Clarificação e concentração

A clarificação e a concentração são processos que têm por finalidade a remoção de alguns contaminantes do plasmídeo com a simultânea redução do volume da solução de lise. A concentração plasmidial geralmente é obtida por precipitação com alguns sais ou álcoois, usualmente o isopropanol ou o etanol (LYDDIATT; O'SULLIVAL, 1998).

A precipitação com polietilenoglicol (PEG) tem sido utilizada, sistemas PEG-8000/NaCl ou $MgCl_2$. Este método baseia-se no fato do tamanho do DNA precipitado ser dependente do massa molar e da concentração polímero (LIS; SCHLEIF, 1975). Sendo assim, pode-se fracionar o DNA de acordo com o seu tamanho ou, simplesmente precipitar todo o DNA (HORN et al., 1995).

Após a concentração do plasmídeo, proteínas, lipopolissacarídios e ácidos nucléicos contaminantes podem ainda ser removidos por precipitação com sais (por exemplo, sulfato ou acetato de amônio) conduzindo a um aumento na pureza do DNA plasmidial (DIOGO et al., 2000).

2.3 Técnicas cromatográficas

A cromatografia é uma das técnicas mais importantes na separação e

purificação de produtos biológicos (PRAZERES et al., 1999), exercendo um papel central na purificação de plasmídeos em larga escala, seja como uma etapa do processo ou como uma ferramenta analítica para o monitoramento dos processos e controle de qualidade (PRAZERES et al., 1998).

Diversos processos cromatográficos têm sido descritos na literatura como forma de purificar ácidos nucléicos, incluindo técnicas de filtração em gel, troca iônica, fase reversa e afinidade (DIOGO, 1999).

Na cromatografia de filtração em gel as moléculas são separadas de acordo com o seu tamanho. Esta técnica permite separar as endotoxinas e RNA dos plasmídeos (HORN et al., 1995; FERREIRA et al., 1997). A lentidão, a baixa resolução, limitação na capacidade (menor do que 10% do volume da coluna para uma boa resolução do produto), diluição do produto final são algumas desvantagens desta técnica (LYDDIATT; O´SULLIVAN, 1998).

Na cromatografia de troca iônica os grupos fosfato do plasmídeo (carregados negativamente) interagem com a fase estacionária da coluna, a qual é carregada positivamente. É necessária a utilização de um gradiente de sal para eluir os ácidos nucléicos, que em princípio devem eluir por ordem do aumento da sua carga total, o que por sua vez é uma função do comprimento da cadeia (PRAZERES et al., 1998). Este método tem a desvantagem de co-purificar justamente com o plasmídeo, DNA genômico, endotoxinas e RNA de alta massa molar devido à semelhante afinidade que estes apresentam para a matriz iônica (DIOGO, 1999).

Green e colaboradores (1997) descreveram um protocolo de cromatografia de fase reversa (CFR) para purificação de plasmídeo em larga escala que utiliza uma coluna de cromatografia líquida de alto desempenho contendo um polímero não poroso e inerte. A adsorção, mecanismo de ligação das moléculas, envolve a interação das moléculas com grupos hidrofóbicos ligados a um material cromatográfico. No entanto, os

suportes usados em CFR possuem uma densidade de ligantes muito superior, portanto a inserção é muito mais forte (PHARMACIA, 1993).

Em 1997, pesquisadores desenvolveram um método de afinidade específico para uma seqüência de DNA para purificação de plasmídeos. Este método baseia-se na formação de uma tripla hélice entre um oligonucleotídeo covalentemente ligado a uma matriz cromatográfica e uma seqüência dupla presente no plasmídeo a ser purificado. As triplas hélices formadas são estáveis apenas para valores ácidos de pH, pelo que a eluição é facilmente conseguida com eluentes básicos. A coluna pode ser reutilizada e permite obter DNA plasmidial purificado. Entretanto, o rendimento mais alto obtido foi de 50% devido às características intrínsecas da própria coluna (WILLS et al., 1997). Além disso, a cinética de formação da tripla hélice é bastante baixa, sendo necessário despender bastante tempo na etapa de ligação (SCHLUEP et al., 1998).

2.3.1 Cromatografia de interação hidrofóbica

A cromatografia de interação hidrofóbica (CIH) é uma técnica cada vez mais utilizada, sobretudo porque exibe características de ligação complementares a outras técnicas, tais como a cromatografia de filtração em gel e de troca iônica (JANSON; RYDÉN, 1993). A cromatografia de interação hidrofóbica apresenta grandes vantagens mediante a diversidade de potenciais condições de eluição que permitem a resolução de misturas complexas que seriam muito difíceis de separar por outros métodos (DIOGO, 1999).

Na cromatografia de interação hidrofóbica procura-se promover a retenção de moléculas de caráter hidrofóbico através da presença de ligantes hidrofóbicos na fase estacionária. A força de interação depende da densidade dos grupos hidrofóbicos à superfície da biomolécula e do tipo e grau de substituição dos ligantes hidrofóbicos ligados à matriz polimérica (JANSON; RYDÉN, 1993).

A cromatografia de interação hidrofóbica foi recentemente descrita por Diogo e colaboradores (2000) para purificação de DNA plasmidial. Esta técnica tem como fundamento a retenção de moléculas de caráter hidrofóbico através da presença de ligantes na fase estacionária. Estas interações têm como base o fato das moléculas de água repelirem os grupos hidrofóbicos, de forma a que estes se juntem, minimizando assim o seu efeito de perturbação na rede de ligações de hidrogênio da água. Usando um eluente de elevada força iônica (de forma a promover este tipo de interação) consegue-se eluir, primeiramente o plasmídeo superenrolado, que não interage com a coluna por ter as suas bases no interior da hélice, e por último o RNA, proteínas, DNA genômico e endotoxinas. Se a força iônica for muito baixa, o plasmídeo fica retido na coluna (DIOGO et al., 2000).

3. EXTRAÇÃO LÍQUIDO-LÍQUIDO

A maioria das técnicas de separação utilizada em processos bioquímicos industriais para a recuperação e isolamento de enzimas, tais como filtração e centrifugação são altamente dependentes do tamanho da partícula (CHAVES, 2000). Os suportes para cromatografia, originalmente desenvolvidos para proteínas, não permitem a entrada do plasmídeo nos poros, sendo, portanto a sua ligação ao suporte apenas superficial (PRAZERES et al., 1998).

A extração líquido-líquido é um processo bem estabelecido na indústria química, incluindo várias aplicações na indústria bioquímica tradicional, como por exemplo, a de antibióticos. A sua utilização tem, no entanto, sido limitada pela baixa compatibilidade entre os materiais de origem biológica e os solventes orgânicos geralmente utilizados, e pelas inerentes dificuldades de validação de um processo que utiliza este tipo de solventes tóxicos (CABRAL et al., 1993).

Porém, o desenvolvimento nas últimas décadas de novas tecnologias de extração usando sistemas de duas fases aquosas têm aberto novas perspectivas para a utilização desta operação unitária. A inserção de etapas de extração líquido-líquido no processo global de produção e purificação de plasmídeos pode, assim, revelar-se extremamente útil no aumento da rentabilidade do produto (RIBEIRO, 2000).

3.1 Sistemas de duas fases aquosas

Em 1896, o microbiologista Beijerinck descreveu a formação de duas fases aquosas macroscópicas quando se misturava uma solução de gelatina (ou amido), agar e água sob certas concentrações. Sendo a fase superior rica em gelatina, e a fase inferior rica em agar. Este fenômeno foi redescoberto por Albertsson que iniciou, há mais de 40 anos, a separação de moléculas biológicas e partículas em sistemas de duas fases aquosas (SDFA). Uma extensa lista destes sistemas foi desenvolvida por diversos pesquisadores e pode ser analisada na Tabela 3.

Desde então, estes sistemas tornaram-se numa poderosa técnica de separação de uma vasta gama de materiais biológicos que incluem plantas, células animais, microrganismos, fungos, vírus, mitocôndrias, proteínas e ácidos nucléicos (HATTI-KAUL, 1999).

Os sistemas inicialmente mais utilizados eram os de polímero/polímero, usando o polietileno glicol (PEG), a dextrana e a metilcelulose (ALBERTSSON, 1986). Em seguida surgiram os sistemas polímero/sal, geralmente com fosfatos, que tinham a vantagem de ser mais simples e ter menores custos. Nestes sistemas a fase superior é constituída majoritariamente por PEG e a fase inferior por sal (SARUBBO, 2000).

Os sistemas de polímeros termoestáveis foram os últimos a serem usados. As soluções destes polímeros, quando aquecidas a uma determinada temperatura, se separam em duas fases; uma enriquecida com o polímero e outra com uma concentração muito

pequena do polímero termoestável (ALRED, 1993).

Os SDFA apresentam grandes vantagens em relação à extração convencional com solventes orgânicos. Os SDFA contêm cerca de 80-95% em água, o que constitui uma característica particularmente interessante na separação de biomoléculas, já que o ambiente é muito pouco agressivo, não alterando a atividade e estabilidade da maioria das biomoléculas. A aplicação destes sistemas permite ainda, tratar grandes volumes num só passo. Os constituintes destes sistemas são de baixa toxicidade. O PEG é biodegradável, foi extensivamente testado na indústria farmacêutica e está registrado em muitos países para fins alimentares (HATTI-KAUL, 1999).

Além disso, a tensão superficial das fases é extremamente baixa o que permite as partículas migrarem livremente entre as duas fases. Sabe-se também que os polímeros têm efeito estabilizante na estrutura e atividade biológica das partículas. Além disso, os SDFA são de fácil aumento de escala e oferecem a possibilidade de adaptar equipamentos e métodos de extração em duas fases (orgânica/aquosa), usadas na indústria química convencional (CUNHA; AIRES-BARROS, 1999).

3.2 Obtenção dos sistemas de duas fases aquosas

Os sistemas de duas fases aquosas são geralmente formados por uma solução aquosa de dois polímeros hidrófilos ou um polímero e de determinados sais. Acima da concentração crítica destes componentes ocorre espontaneamente a separação de fases, predominando um ou outro componente em cada uma das fases resultantes (ZASLAVSKY, 1995).

Cada SDFA pode ser caracterizado por um único diagrama de fases, que contém a composição das fases em equilíbrio para o sistema. Os dados fundamentais para qualquer tipo de processo de extração líquido-líquido são as composições de equilíbrio das fases (DIAMOND; HSU, 1992). A literatura descreve diagramas de fases para diferentes

sistemas (ALBERTSSON, 1986; ZASLAVSKY, 1995).

Tabela 3. Exemplos de sistemas de duas fases aquosas, adaptado de SEBASTIÃO et al., 1996.

Componente 1	Componente 2	Referência
Polietileno glicol	Dextrana	Albertsson, 1986
Polietileno glicol	Hidroxipropilamido (AquaphasePPT*, Reppal* PES, preparações utilizadas nas indústrias do papel, alimentar e têxtil)	Tjerneld et al., 1986 Buitelaar et al., 1992 Venâncio et al., 1993
Polietileno glicol	Fosfato de potássio	Albertsson, 1986
Polietileno glicol	Sulfato de potássio	Albertsson, 1986
Polietileno glicol	Sulfato de magnésio	Eitman; Gainer, 1990
Polietileno glicol	Sulfato de amônio	Yang et al., 1995
Polietileno glicol	Policaju	Sarubbo et al., 2000
Ficol	Dextrana	Albertsson, 1986
Álcool polivinílico	Co-polímeros acrílic	Hughes; Lowe, 1988
Co-polímeros não-iônicos de óxido de etileno e óxido de propileno	Dextrana/hidroxipropilmido	Alred et al., 1994 Modlin et al., 1994
Detergentes não iônicos baseados no polioxietileno (Triton X, Triton N, Tween)	-	Sánchez-Ferrer et al., 1994

*São designações comerciais do hidroxipropilamido

A Figura 2 mostra um diagrama de fases para um sistema polímero e sal. A concentração do polímero P é representada em ordenada e a concentração do sal em abscissa; as concentrações são expressas como percentual (massa/massa). A linha curva separando as duas áreas é chamada binodal. Todas as misturas que têm composições representadas pelos pontos acima da linha são pontos de duas fases e os pontos abaixo representam uma solução homogênea (ZASLAVSKY, 1995).

A linha que une dois pontos sobre a binodal para um determinado sistema designa-se por linhas de amarração (tie line). Qualquer ponto sobre a mesma tie line resulta num sistema em que as fases têm a mesma composição, mas diferentes volumes. Por exemplo, em um sistema com concentração total representada por A, correspondem duas fases em equilíbrio cuja composição é dada pelos pontos B (fase inferior) e C (fase superior). Como a densidade das fases é muito próxima da água, a razão dos volumes pode ser obtida, aproximadamente, a partir das distâncias AB e AC no diagrama. O comprimento da tie line depende da concentração total do sistema, representando uma medida da diferença entre as fases em equilíbrio. À medida que o comprimento da tie line diminui (C'B'), o sistema aproxima-se do ponto crítico (K), isto é, ponto na binodal em que os volumes e composições das duas fases teoricamente se tornam iguais (RIBEIRO, 2000).

3.3 Termodinâmica do diagrama de fases

A separação das fases nos SDFA pode ser analisada sob duas importantes abordagens. A teoria desenvolvida por Flory-Huggins sugere que o enorme aumento de entalpia associado à mistura das longas cadeias de dois polímeros sobrepõe-se à perda de entropia gerada pela criação de duas fases distintas, ou seja, é mais desfavorável a mistura do que a separação de fases (HUDDLESTON et al., 1991). Sendo assim, quanto

maior for o polímero mais fácil será a separação e, portanto, menor a concentração mínima à qual esta ocorre (ALRED, 1993).

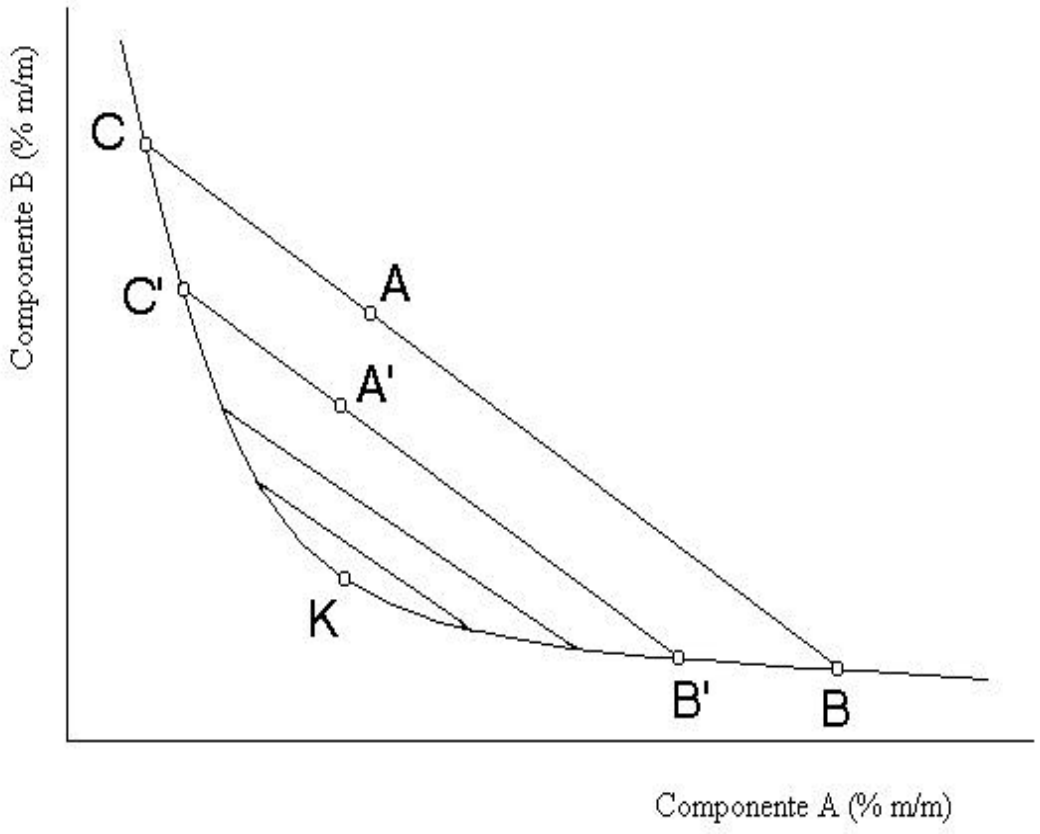

Figura 2. Diagrama de fases de um sistema de duas fases aquosas com os componentes A e B.

Por outro lado, Zaslavsky (1995) afirma que a estrutura da água também deve ser considerada na separação de fases. As moléculas de PEG são homopolímeros constituídos por repetições de resíduos de etileno ligados por um átomo de oxigênio [HO-($HH_2CH_2O)_n$-CH_2CH_2OH]. As moléculas de água podem, portanto, formar ligações de hidrogênio com este átomo. O PEG está assim rodeado por uma camada de hidratação estando as zonas hidrofóbicas blindadas nos poros da matriz de polímero. Outro polímero, ou um sal, terá um padrão de hidratação diferente. Esta diversidade na

orientação das moléculas de água é capaz portanto, de provocar uma repulsão entre os diferentes componentes dos sistemas, levando assim à formação das duas fases (RIBEIRO, 2000).

A partição de biomoléculas em sistemas de duas fases aquosas resulta do somatório de uma série de forças que atuam nos sistemas. As forças das quais depende a partição resultam, por um lado, da composição e interações existentes em cada fase e, por outro, da interação entre o soluto e as fases do sistema. Em relação à composição das fases o volume livre e as interações, favoráveis ou desfavoráveis, são determinantes entre os seus componentes (JOHANSSON et al., 1998).

3.4 Fatores que influenciam o comportamento das fases

O diagrama de fases de um determinado sistema pode ser influenciado por diversos fatores, incluindo a concentração e a massa molar dos polímeros, pH, temperatura e adição de sais, embora os efeitos e os mecanismos pelo quais estes influenciam a separação das fases ainda não estejam completamente elucidados (DIAMOND; HSU, 1992).

3.5 Fatores que influenciam a partição de biomoléculas em sistemas de duas fases aquosas

Os fatores inerentes ao próprio sistema podem ser: escolha dos componentes do sistema, massa molar do polímero, concentração do polímero e de sais, tipos de íon presentes, (força iônica e pH) e espécie da biomolécula a sofrer a partição: massa molar, carga, hidrofobicidade, conformação, presença de ligantes bioespecíficos. A seleção de propriedades dos sistemas de fases apropriada para purificação de uma biomolécula específica é ainda empírica, embora existam regras gerais com relação ao efeito das características do polímero e composição iônica da biomolécula a sofrer partição (CASCONE et al., 1991).

Do ponto de vista das propriedades físico-químicas da biomolécula, o coeficiente de partição, K, definido como a razão entre a concentração de biomolécula na fase superior e inferior, pode ser traduzido por vários parâmetros, fornecendo a expressão:

$$\ln K = \ln K_{hidrof} + \ln K_{el} + \ln K_{bioesp} + \ln K_{cof} + \ln K_{tamanho}$$

onde: $\ln K_{hidrof}$, $\ln K_{el}$, $\ln K_{bioesp}$, $\ln K_{cof}$, $\ln K_{tamanho}$ expressam a contribuição, para o coeficiente de partição total (K), a natureza hidrofóbica, força eletrostática, bioespecididade, conformação e tamanho da molécula, respectivamente. Estes parâmetros podem ser manipulados a fim de atingir a partição ótima da biomolécula (PORTO, 1998).

A partição de uma biomolécula em sistema de duas fases tendo o PEG como fase superior aumenta com a diminuição da massa molar do PEG; este fato é tanto mais pronunciado quanto maior for a massa molar da molécula biológica a sofrer a partição (ALBERTSSON et al., 1987).

A adição de sais, mesmo em concentrações milimolares, influencia fortemente a partição de materiais carregados eletricamente. Embora os sais se distribuam quase que igualmente entre as fases, existem pequenas, mas significantes diferentes afinidades pelas fases, criando uma diferença de potencial elétrico entre as fases, que por sua vez direciona a partição de materiais biológicos carregados. A influência de diferentes sais na partição de proteínas a baixas concentrações foi estudada no sistema PEG-dextrana (ALBERTSSON, 1986).

A alteração do pH do sistema contendo sal poderá alterar a partição pela mudança na carga da biomolécula. Como a força iônica na maioria dos materiais biológicos é dependente do pH, a escolha deste e de um sal pode constituir um modo efetivo de ajuste da partição (JOHANSSON; JOELSSON, 1985).

O fato da partição depender de um número grande de fatores distintos confere

considerável versatilidade aos sistemas de duas fases aquosas na separação de misturas de componentes. Entretanto, a existência de muitas variáveis, na sua maioria interdependentes, torna extremamente difícil a previsão teórica do coeficiente de partição de um determinado soluto, obrigando por vezes a um trabalho experimental exaustivo (SEBASTIÃO et al., 1996).

3.6 Partição de ácidos nucléicos

Desde o início dos anos 60 têm sido descritos fatores que influenciam a partição de DNA em sistemas de duas fases aquosas (LIF et al., 1961; FRICK; LIF, 1962; ALBERTSSON, 1962; MULLER, 1985). No entanto, a grande maioria dos estudos realizados sobre a partição de DNA foi realizada em sistemas polímero/polímero e com DNA linear ou cromossômico. Estudos de partição de DNA plasmidial em sistemas PEG/sal são muito escassos (OHLSSON et al., 1978; COLE, 1991; RIBEIRO et al., 2000).

Em 1962, Albertsson descreveu a partição de DNA cromossômico e do fago T2 em sistemas PEG/dextrana, sendo o DNA particionado para a fase superior a baixas concentrações de tampão (0,1-0,15 M), o DNA passou a ser transferido para a fase inferior. Isto parece ser característico destes sistemas e deve-se provavelmente às características polieletrolíticas das substâncias distribuídas.

O uso de sais de forma a direcionar a partição de uma molécula é muito comum nos SDFA. Os ácidos nucléicos são extremamente sensíveis a alterações na composição iônica (ALBERTSSON, 1986). Esta extrema sensibilidade pode ser explicada pela presença de um grande número de grupos fosfato na superfície do DNA. A preferência de cada íon para uma das fases e o fato deste não poder particionar independentemente, uma vez que cada fase tem que ser eletroneutra no equilíbrio, cria uma tensão que se manifesta como uma diferença de potencial entre as fases (PFENNING et al., 1998).

A estrutura primária e secundária do DNA influencia consideravelmente a sua partição. Através de poliribonucleotídeos de cadeia simples estudou-se a influência das bases em sistemas PEG/dextrana. Os nucleotídeos polipurínicos têm um K menor que os polipirimidínicos segundo a ordem: poli(U) > poli(C) > poli(A) > poli(G) (MULLER, 1985).

O efeito da estrutura secundária é muito mais acentuado. A desnaturação pela temperatura da cadeia dupla de DNA, seguida de rápido resfriamento para evitar a renaturação, permitiu a separação das cadeias desnaturadas que se particionam para a fase inferior a baixas forças iônicas. As razões apontadas para este fato foram a mudança na configuração do DNA (ALBERTSSON, 1962). Estes sistemas podem ser úteis na verificação da integridade do DNA (RIBEIRO, 2000).

A concentração dos polímeros pode inverter a preferência do DNA por uma das fases, para uma mesma força iônica. Estudos em sistemas PEG/dextrana a baixa força iônica (0,005 M de tampão) revelaram que um aumento da concentração dos polímeros leva a uma diminuição do valor de K. Um aumento de 3,5% PEG/5% dextrana (p/p) para 6% PEG/8% dextrana causou uma alteração no K de cadeias poli-U de 4,4 para 0,02, ou seja o ácido nucléico passou da fase superior para a inferior (ALBERTSSSON, 1965).

Como era previsível pela influência da estrutura e do ambiente no K, também a ligação de moléculas aos ácidos nucléicos pode variar o seu comportamento em SDFA. As maiores diminuições no valor de K foram observadas com a ligação ao DNA das proteínas quimiotripsina e RNA polimerase (DNA dependente), que diminuem em cerca de três ou quatro ordens de grandeza o coeficiente de partição (MULLER, 1985).

4. OBJETIVOS

4.1 Objetivo Geral

Produzir e purificar DNA plasmidial a partir de *Escherichia coli* recombinante.

4.2 Objetivos Específicos

Analisar a influência das condições de cultivo (meio de cultura, velocidade de agitação e concentração de antibiótico) na produção de DNA plasmidial;

Verificar a estabilidade plasmidial em diferentes condições de cultivo;

Investigar o efeito da partição de DNA plasmidial e RNA no sistema de duas fases aquosas PEG/Sal;

Verificar a partição de proteínas totais no sistema de duas fases aquosas PEG/Sal;

Avaliar o efeito do volume do lisado celular na recuperação do DNA plasmidial e

Purificar DNA plasmidial através de cromatografia de interação hidrofóbica.

5. REFERÊNCIAS BIBLIOGRÁFICAS

ALBERTSSON, P. A.; CAJARVILLE, A.; BROOKS, D. E.; TJERNELD, F. **Partition of proteins in aqueous polymer two-phase systems and the effect of molecular weight of the polymer**. Biochimica et Biophysica Acta, v. 926, p. 87-93, 1987.

ALBERTSSON, P. A. **Partition of cells particles and macromolecules**. 3 ed. Willey, New York, 1986.

ALBERTSSSON, P. A. Partition studies on nucleic acids. **Influence of electrolytes, polymer concentration and nucleic acid conformation on the partition in the dextran-polyethulene glycol system.** Biochimica et Biophysica Acta, v. 103, p. 1-12, 1965.

ALBERTSSON, P. A., **Partition of double-stranded and single-stranded deoxyribonucleic acid.** Archives of Biochemistry and Biophysics, Supplment 1, p. 264-270, 1962.

ALRED, P. A.; KOZLOWSKI, A.; HARRIS, M.; TJERNELD, F. **Application of temperature-induced phase partitioning at ambient temperature for enzyme purification.** Journal of Chromatography A, v. 659, p. 289-298, 1994.

ALRED, P. A. **Biomolecule purification using temperature-induced phase separation and affinity partitioning**. Tese de doutorado, Universidade de Lund, Suécia, 1993.

BAHERI, H. R.; HILL, G. A.; ROESLER, W. J. **Modelling plasmid instability in batch and continuous fermentors.** Bichemical Engineering Journal, v. 8, p. 45-50, 2001.

BEIJERICK, M. W. **Bakterio Parasitenkd Infektionskr**, v. 2, 627-689, 1896.

BOLES, T. C.; WHITE, J. H.; COZZARELLI, N. R. **Structure of plectonemically supercoiled DNA**. Journal Molecular Biology, v. 213, p. 931-951, 1990.

BROWN, T. A. **Genética um enfoque molecular**. 3 ed, Editora Guanabara Koogan S. A. Rio de Janeiro, RJ, 1999.

BUITELAAR, R. M.; LEENEM, E. J. T. M.; TRAMPER, J. **Growth and secondary metabolite production by hairy roots of *Tagetes patula* in aqueous two-phase systems**. Biocataysis, v. 6, p. 73-80, 1992.

CABRAL, J. M. S.; AIRES-BARROS, M. R. **Liquid-liquid extraction of biomolecules using aqueous two-phase systems**: in Recovery process for biological materials, John Wiley & Sons ltd (editores), New York, p. 273-301, 1993.

CARLSON, A.; SIGNS, M.; LIERMANN, L.; BOOR, R.; JEM K. J. **Mechanical disruption of *Escherichia coli* for plasmid recovery**. Biotechnology Bioengineering, v. 48, p. 303-315, 1995.

CASCONE, O.; ANDREWS, B. A.; ANSEJO, J. A. **Partitioning and purification of thaumatin in aqueous two-phase systems**. Enzyme Microbiology Technology, v. 13, p. 629-635, 1991.

CHAVES, A. C. **Produção e purificação de proteína recombinada de *Schistosoma mansoni* utilizando sistemas de duas fases aquosas**. Tese de doutorado, Universidade Técnica de Lisboa, Instituto Superior Técnico, Lisboa, 2000.

CICCOLINI, L. A. S.; SHAMLOU, P. A.; TITCHENER-HOOKER, N. J.; WARD, J. M.; DUNNILL, P. **Time course of SDS-alkaline lysis of recombinant bacterial cells for plasmid release**. Biotechnology and Bioengineering, v. 6, p. 768-770, 1998.

COLE, K. D. **Purification of plasmid and high molecular mass DNA using PEG-salt two-phase extraction.** Biotechniques, v. 11, p. 18-24, 1991.

COSTA, S. O. P. **Genética molecular e de microorganismos** – Os fundamentos da engenharia genética. Editora Manole, São Paulo, SP, 1987.

CUNHA, T.; AIRES-BARROS, R. **Large-scale extraction of proteins**. Aqueous two-phase systems: methods and protocols. Rajni Hatti-Kaul (editor), Humana Press, New Jersey, 1999.

DIAMOND, A. D.; HSU, J. T. **Aqueous two-phase systems for biomolecule separation**. Advances Biochemistry Engineering, v. 47, p. 89-135, 1992.

DIOGO, M. M. F. R. **Purificação de plasmídeos para terapia gênica por cromatografia de interação hidrofóbica**. Dissertação de mestrado, Universidade Técnica de Lisboa, Instituto Superior Técnico, Lisboa, 1999.

DIOGO, M. M.; QUEIROZ, J. A.; MONTEIRO, G. A.; FERREIRA, G. N. M.; MARTINS, S. A. M.; PRAZERES, D. M. F. **Purification of a cystic fibrosis vector for gene therapy using hydrophobic interction chromatography**. Biotechnology and Bioengineering, v. 68, p. 576-583, 2000.

EITEMAN, M. A.; GAINER, J. L**. Peptide hydrophobicity and partitioning in polyethylene glycol-magnesium sulfate aqueous two-phase systems**. Biotechnology Progress, v. 6, p. 479-484, 1990.

FERREIRA, G. N. M.; MONTEIRO, G. A.; PRAZERES, D. M. F.; CABRAL, J. M. S. **Downstream processing of plasmid DNA for gene therapy and DNA vaccine applications**. Tibtech., v. 18, p. 380-386, 2000.

FERREIRA, G. N. M.; CABRAL, J. M. S.; PRAZERES, D. M. F. **A comparison of gel filtration chromatographic supports for plasmid purification**. Biotechnology Techniques, v. 11, p. 417-420, 1997.

FRICK, G.; LIF, T. **Relation between size and distribution of DNA molecules in a two-phase polymer system.** Archives of Biochemistry and Biophysics. v. 1, p. 271-275, 1962.

GANUSOV, V. V.; BRILKOV, A. **Estimating tje instability parameters of plasmid-bearing cells. I. Chemostat culture**. Journal of Theoretical Biology, v. 219, p. 193-

205, 2002.

GREEN, A. P.; PRIOR, G. M.; HELVESTON, N. M.; TAITTINGER, B. E.; LIU, X.; THOMPSON, J. A. **Preparative purification of supercoiled plasmid DNA for therapeutic applications**. BioPharm, v. 5, p. 52-62, 1997.

HATTI-KAUL, R. Aqueous two-phase systems. Hatti-Kaul, R. (editor). Methods in biotechnology - **Aqueous two-phase systems**: Methods and protocols. Human Press: New Jersey, v. 11, p. 1-10, 1999.

HORN, N. A.; MEEK, J. A.; BUDAHAZI, G.; MARQUET, M. **Cancer gene therapy using plasmid DNA**: purification of DNA for human clinical trials. Human Gene Therapy, v. 6, p. 565-573, 1995.

HUDDLESTON, J.; VEIDE, A.; KOHLER, K.; FLANAGAN, J.; ENFORS, S-O.; LYDDIATT, A. **The molecular basis of partitioning in aqueous two-phase systems**. Trends Biotechnolgy, v. 9, p. 391-388, 1991.

HUGHES, P.; LOWE, C. R. **Purification of proteins by aqueous two-phase partition in novel acrylic co-polimer systems.** Enzyme Microbiology Technology, v. 10, p. 115-122, 1988.

JANSON, J. C.; RYDÉN, L. **Protein separation and purification.** Biotechnology. v. 3. Rehm, H. –J & Reed, G. (editores), 617-642, 1993.

JIMINEZ, A.; DAVIES, J. **Expression of a transposable antibiotic resistance element in *Saccharomyces*.** Nature, v. 287, p. 869-871, 1980.

JOHANSSON, G.; JOELSSON, M. **Preparation of Cibracron blue F3GA (polyethylene glycol) in large scale for use affinity partitioning**. Biotechnology and Bioengineering, v. 27, p. 621-625, 1985.

JOHANSSON, H. O.; KARLSTROM, G.; TJERNELD, F.; HAYNES, C. A. **Driving forces for phase separation and partitioning in aqueous two-phase systems.** Journal Chromatography B, v. 718, p. 3-17, 1998.

LEVY, M. S.; O´KENNEDY, R. D.; AYAZI-SHAMLOU, P.; DUNNILL, P. **Biochemical engineering approaches to the challenges of producing pure plasmid DNA.** Tibtech, v. 18, p. 296-305, 2000.

LIF, J. T.; FRICK, G.; ALBERTSSON, P. A. **Fractionation of nucleic acids in aqueous polymer two-phase systems.** Journal of Molecular Biology, v. 3, p. 1701-1717, 1961.

LIS, J. T.; SCHLEIF, R. **Size fractionation of double-stranded DNA by precipitation with polyethylene glycol.** Nucleic Acids Research. v. 2, p. 383-389, 1975.

LYDDIATT, A.; O'SULLIVAN, D. A. **Biochemical recovery and purification of gene therapy vectors**. Current Opinion in Biotechnology, v. 9, p. 177-185, 1998.

McFAIL-ISOM, L.; SINES, C. C.; WILLIAMS, L. D. **DNA structure: cations in charge?** Current Opinion in Structural Biology, v. 9, p. 298-304, 1999.

MODLIN, R. F.; ALRED, P. A.; TJERNELD, F. **Utilization of temperature induced phase separation for the purification of ecdysone and 20-hydroxycedsone from spinach.** Journal Chromatography A, v. 668, p. 229-236, 1994.

MÔO-YOUNG, M.; CHISTI, Y.; ZHANG, Z.; GARRIDO, F.; BANERJEE, U.; VLACH, D. **Bioprocessing with genetically modified and other organisms: case studies in processing constraints**. Annals of the New York Academic of Sciences, v. 782, p. 391-401, 1996.

MULLER, W. **Partitioning of nucleic acids. In: Partitioning in aqueous two-phase systems: theory, methods, uses and application to biotechnology**. H. Walter; D. E. Brooks; D. Fischer (editores). Academic Press, Orlando, Fla., pp. 227-266, 1985.

OHLSSON, R.; HENTSCHEL, C. C.; WILLIAMS, J. G. **A rapid method for the isolation of circular DNA using an aqueous two-phase partition system.** Nucleic Acids Research, v. 5, p. 583-590, 1978.

O'KENNEDY, R. D.; BALDWIN, C.; KESHAVARZ-MOORE, E. **Effects of**

growth medium selection on plasmid DNA production and initial processing steps. Journal of Biotechnology, v. 76, p. 175-183, 2000.

OLD, R. W.; PRIMROSE, S. B. **Principles of gene manipulation: an introduction to genetic engineering**, Blackwell Science Inc, 1981.

PFENNING, A.; SCHWERING, A.; GAUBE, J. **Consistent view of electrolytes in aqueous two-phase systems**. Journal of Chromatography B, v. 711, p. 45-52, 1998.

PHARMACIA. **Hydrophobic interaction chromatography:** principles and methods. Uppsala: Pharmacia Bioprocess Technology, 1993.

PORTO, A. L. F. **Extração líquido-líquido de proteínas utilizando sistemas de duas fases aquosas em coluna de discos perfurados rotativos.** Tese de doutorado, Universidade de Campinas, Campinas, 1998.

PRAZERES, D. M. F.; FERREIRA, G. N. M.; MONTEIRO, G. A.; COONEY, C. L.; CABRAL, J. M. S. **Large-scale production of pharmaceutical-grade plasmid DNA for gene therapy, problems and bottlenecks.** Trends Biotechnology, v. 17, p. 169-174, 1999.

PRAZERES, D. F. M.; SCHUEP, T.; COONEY, C. L. **Preparative purification of supercoiled plasmid DNA using anion-exchange chromatography.** Journal of Chromatography A, v. 806, p. 31-45, 1998.

PROCTOR, G. N. **Mathematics of microbial plasmid instability and subsequent differential growth of plasmid-free and plasmid-containing cells, relevant to the analysis of experimental colony number data**. Plasmid, v. 32, p. 101-130, 1994.

RIBEIRO, S. C.; MONTEIRO, G. A.; CABRAL, J. M. S.; PRAZERES, D. M. F. **Isolation of plasmid DNA from cell lysates by aqueous two-phase systems.** Biotechnology and Bioengineering, v. 78, p. 376-384, 2002.

RIBEIRO, S. C. A. D. **Extracção de DNA plasmídico por sistemas de duas fases aquosas.** Dissertação de mestrado, Universidade Técnica de Lisboa, Instituto

Superior Técnico, Lisboa, 2000.

RIBEIRO, S. C.; MONTEIRO, G. A.; MARTINHO, G.; CABRAL, J. M. S.; PRAZERES, D. M. F. **Quantitation of plasmid DNA in aqueous two-phases systems by fluorescence analysis.** Biotechnology Letters, v. 22, p. 1101-1104, 2000.

SANCHEZ-FERRER, A.; PEREZ-GILABENT, M.; NÚNUEZ, E.; BRU, R.; GARCI-CARMONE, F. **Triton-114 phase implant protein purificatin.** Journal of Chromatography A, v. 668, p. 75-83, 1994.

SARUBBO , L. A.; OLIVEIRA, L. A.; PORTO, A. L. F.; DUARTE, H. S.; CARNEIRO-LEÃO, A. M. A.; LIMA-FILHO, J. L.; CAMPOS-TAKAKI, G. M.; TAMBOURGI, E. B. **New aqueous two-phase system based on cashew-nut tree gum and poly(ethylene glycol).** Journal of Chromatography B, v. 743, p. 79-84, 2000.

SCHLEEF, M. **Issues of large-scale plasmid DNA manufacturing.** In; H.-J. Rehm & G. Reed (eds). Biotechnology. Wiley-VCH, Weinheim, v. 5, pp. 443-469, 1999.

SCHLICK, T.; OLSON, W. K. **The influence of salt on the structure and energetics of supercoiled DNA.** Biophysical Journal, v. 67, p. 2146-2166, 1994.

SCHLUEP, T.; COONEY, C. L. **Purification of plasmids by triplex affinity interation.** Nucleic Acids Research, v. 26, p. 4524-4528, 1998.

SEBASTIÃO, M.J.; CABRAL, J.M.S.; AIRES-BARROS, M.R. **Improved purification protocol of a *Fusarium solani pisi* recombinant cutinase by phase partitioning in aqueous two-phase systems of polyethylene glycol and phosphate.** Enzyme Microbiology Technology, v. 18, p. 251-260, 1996.

SINDEN, R. R. **DNA structure and function**. Academic Press, San Diego, 1994.

THEODOSSIOU, I.; COLLINS, I. J.; WARD, J. M., THOMAS, O. R. T.; DUNNILL, P. The processing of a plasmid-based gene from *Escherichia coli*. Primary recovery by filtration. Bioprocess Engineering, v. 16, p. 175-183, 1997.

TJERNELD, F.; BERNER, S.; CAJARVILLE, A.; JOHANSSON, G. **New aqueous**

two-phase system based on hydroxypropil starch useful in enzyme purification. Enzyme Microbiology Technology, v. 8, p. 417-423, 1986.

VENÂNCIO, A.; TEIXEIRA, J. A.; MOTA, M. **Evaluation of crude hydroxypropil starch as a bioseparation aqueous-phase forming polymer.** Biotehnology Progress, v. 9, p. 635-639, 1993.

VYAS, V. V.; GUPTA, S.; SHARMA, P. **Stability of a recombinant shutlle plasmid in *Bacillus subtilis* and *Escherichia coli*.** Enzyme Microbiology Technology, v. 16, p. 240-246, 1994.

WANG, Z.; LE, G.; SHI, Y.; WE, G. **Medium design for plasmid DNA production based on stoichiometric model.** Process Biochemistry, v. 36, p. 1085-1093, 2001.

WILLS, P.; ESCRIOU, V.; WARNEY, A.; LACROIX, F.; LAGNEAUX, D.; OLLIVIER, M.; CROUZET, J.; MAYAUX, J. F.; SCHERMAN, D. **Efficient purification of plasmid DNA for gene transfer using triple-helix affinity chromatography.** Gene Therapy, v. 4, p. 323-330, 1997.

YANG, W. Y.; LIN, C. D.; CHU, I. M.; LEE, C. J. **Extraction of cephalosporin C from whole broth and separation of desacetyl cephalosporin C by aqueous two-phase partition.** Biotechnology and Bioengineering, v. 43, p. 439-445, 1995.

ZASLAVSKY, B. Y. **Aqueous two-phase partitioning: Physical chemistry and bioanalytical application.** New York: Marcel Dekker, 1995.

ZHANG, Z.; MOO-YOUNG, M.; CHISTI, Y. **Plasmid stability in recombinant *Saccharomyces cerevisiae*.** Biotechnology Advances, v. 14, p. 401-435, 1996.

6. CAPÍTULOS

Capítulo 1 - Effect of cultural conditions on plasmid DNA production and stability

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Effect of cultural conditions on plasmid DNA production and stability

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Effect of cultural conditions on plasmid DNA production and stability

Abstract

Cultures of recombinant *Escherichia coli* containing the plasmid pD2 were grown in two medium TB (Terrific broth) or LBG (Luria Bertani with glucose). Three velocity of agitation (120, 160 and 200 rpm) and five kanamycin concentrations (10, 20, 30, 40 and 50 μg mL^{-1}) were the parameters studied for to assess their effects of cultural conditions on plasmid DNA production and stability for plasmid-based gene therapy. The velocity of agitation that provided highest results in relationship biomass and plasmid DNA production was 200 rpm. While the concentrations 30 to 50μg mL^{-1} of kanamycin provided similar results for all the studied conditions and the culture medium TB medium which showed of better results for the biomass production and DNA. The plasmid was (82%) stable in TB medium.

Keywords: culture conditions, growth rate, plasmidial DNA, production.

Introduction

The success of any recombinant-based fermentation is combination of interaction between the fermentation environmental, the host organism and its recombinant genetic elements (O' Kennedy et al., 2000). Plasmids are popular vehicles for introducing new genes into living cells. Many commercial plasmid vectors are available that can be used to carry a desired gene into a host organism (Baheri et al., 2001). Plasmid DNA has recently acquired considerable interest to its attractive potential application in gene therapy and DNA vaccines (Levy et al., 2000; Ferreira et al., 2000; Wang et al., 2001; Ribeiro et al., 2002). Plasmid instability in recombinant cultures is often a serious problem as it reduces the overall levels of the desired product in the process and thus has a negative impact on the economics of the bioprocess (Gupta et al., 1995). Recombinant plasmid can be lost from cells due to defective segregation of plasmid during cell division or structural instability of the plasmid material due to mutation (Baheri et al., 2001). A number of experimental studies have demonstrated that plasmid stability is affected by plasmid partitioning, growth media, growth rate, plasmid copy number, recombination backgrounds of the host and size of the insert (Gupta et al., 1995). The instability of a recombinant plasmid in a microbial culture may reduce the overall levels of the desired product in the cultivation, have a negative impact on specific activities of proteins, and increase the production costs, since growth substrates are consumed by nonproductive cells that may have a significant growth rate advantage over the cells harboring intact recombinant plasmid (Vyas, 1994). *Escherichia coli* is most frequently used host for the industrial production of recombinant DNA technology-based proteins, offers advantage in that it has the best understood genetic and physiological systems, fast growth, easy transformation, and a very large number of vectors available (Gupta et al., 1995). However, across different species, complex media tend to reduce growth rate-associated plasmid instability

while defined media tends to alleviate segregation rate-associated plasmid instability (O' Kennedy et al., 2000). In this paper we have studied the effects of two culture media using different agitation speeds and kanamycin concentrations on the production of the pD2 plasmid.

Materials and methods

Plasmid and bacterial strains

The plasmid used in this work was pD2, a dengue 2 plasmid DNA vaccine expressing the pre-membrane and envelope proteins (pre M-E), a plasmid of 4.5 Kb with an kanamycin (Sigma, St. Louis, MO, USA) resistant marker (Lu et al., 2003). It was transformed into *Escherichia coli* XL1 Blue. The host was maintained in 25% (v/v) glycerol at –70ºC. Details of the techniques used to introduce plasmid into the host strain are given elsewhere (Sambrook et al., 1989).

Innoculum preparation

The recombinant *E. coli* was grown overnight (18h) in 50 mL flask containing 10 mL of Luria-Bertani (LB) medium containing tryptone (10 g L^{-1}), yeast extract (5 g L^{-1}) and NaCl (10 g L^{-1}) supplemented with 30 µg mL^{-1} of kanamycin in an orbital shaker at 160 rpm and 37ºC.

Culture conditions

All fermentation were carried out in 250 mL flasks containing 50 mL of Terrific Broth (TB) medium containing tryptone (20 g L^{-1}), yeast extract (24 g L^{-1}), glycerol (0.4% v/v), KH_2PO_4 (0.017M), K_2HPO_4 (0.072M) or LBG medium containing glucose (10 g L^{-1}), tryptone (10 g L^{-1}), yeast extract (5 g L^{-1}) and NaCl (10 g L^{-1}). Both media

were supplemented with five different kanamycin concentrations (10, 20, 30, 40 and 50 μg mL $^{-1}$). The innoculum size used was 10% of the culture volume from an overnight culture. The flasks were incubated in an orbital shaker at 120, 160 and 200 rpm for 8 hours at 37ºC.

Determination of dry cell weight (DCW)

Aliquots (10 mL) of culture fluid were centrifuged at 15,000 g for 10 min at 4ºC in pre-weighed glass. The supernatant was decanted and cells were resuspended in an equal volume of sterile reverse osmosis H_2O and centrifuged again. The supernatant was decanted and the cell pellets were dried to a constant weight overnight in 105ºC oven.

Measurement of plasmid stability

The bacterial culture samples were diluted appropriately in physiological saline (0.9% w/v NaCl), plated onto LB-agar, and incubated at 37ºC for 18 h. Hundred and fifty colonies were replica plated onto LB-agar and LB-agar containing kanamycin (30 μg mL $^{-1}$) and incubated 18-24h. The number of colonies growing on LB-agar, but not on LB-kanamycin agar represented the proportion of plasmid-containing cells.

Preparation of the total and plasmid DNA

A modified alkaline method was applied for cell lysis (Sambrook et al., 1989). Cells (50mL) were harvested by centrifugation at 15,000 x g (20 min, 4ºC) and the pellets resuspended in 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0. The cells were lysed by adding and gently mixing (10 min on ice) of 200 mM NaOH, 1% (w/v) sodium dodecyl sulphate. The lysate was neutralized with a solution of 3M potassium acetate, 11.5% (v/v) glacial acetic acid (10 min on ice). This neutralized lysate was clarified by centrifugation at 12,000 x g for 30 min. The supernatant was

precipitated with 0.7 vols isopropanol (45 min at 4ºC). Pellets were obtained by centrifugation at 10,000 x g (for 20 min at 4ºC) and then redissolved in 10mM TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0), while for the preparation of plasmid DNA it was used the Pharmacia purification mini kit, resuspended in TE buffer.

DNA determination

Total and plasmid DNA determination can be carried out using a spectrophotometer (Model 3000, Pharmacia). Concentration of total and plasmid DNA was calculated from the absorbance at 260 nm (A_{260}) (an A_{260} of 1 the corresponds to a 50 μg mL^{-1} double stranded (ds) DNA solution). Purity of the samples was checked by the ratio of absorbance at 260 and 280 nm.

Statistical analysis

An analysis of variance for culture medium, agitation velocity and kanamycin concentrations were carried out and treatment effects were evaluated by using F test statistic ($P<0.05$). These variance analyses were carried out with the software Statistic (Statsoft, Inc., Tulsa, OK). The standard error SE ($P<0.05$) was estimated and the comparison of the treatments was averages carried out by adjustment of the best polynomial.

Results and discussion

Biomass and plasmid production

The velocity of biomass production of a recombinant *Escherichia coli* XL1 Blue grown in two different media, five concentrations of kanamycin and three agitation of velocity are demonstrated in Figure 1. This figure shows the statistical analysis of the

biomass production variance data. They indicate that for all the kanamycin concentrations used in the culture medium the results were significantly the same. The TB culture medium demonstrated, for all the analyzed velocities, that the biomass production was higher when compared with LBG medium. Biomass production is a function of nutrient supplies and is affected by environmental factors, such temperature, pH and aeration. Recombinant fermentation processes aim at large-scale production, high product yield, high selectivity and low cost of raw materials. The strategies used aim to keep high growth rates assuring high cell density and high levels of product (Chaves et al., 1999). Economic large-scale plasmid production from *E. coli* requires the concomitant optimization of plasmid copy number (specific yield) and of biomass concentration (Swartz, 2001). Production velocity of plasmid DNA is shown in Fig. 2. The best results for DNA plasmidial production were found for concentrations of 30, 40 and 50µg mL $^{-1}$ of kanamycin at 200 rpm (230µg mL $^{-1}$ h^{-1}) in TB medium. For LBG the concentrations of 40 and 50µg mL $^{-1}$ of kanamycin demonstrated similar behavior among the three-used velocity of agitation and were smaller when compared to velocity production in TB medium. Studies accomplished by Wang et al. (2001) for the medium design goes plasmid DNA production based on stoichiometric model showed best results with defined MW1 medium (60.0mg L $^{-1}$). O´Kennedy et al. (2000), verifying the effect of growth medium selection on plasmid DNA production and initial processing steps obtained plasmid yield of 0.56mg L $^{-1}$. The plasmid DNA specific production is demonstrated in Fig. 3 for the different cultivation conditions tested. The best DNA specific production rates were obtained using TB medium in the concentrations of 30, 40 and 50µg mL $^{-1}$ of kanamycin for the velocity of 200 rpm. With the increase of the velocity, the specific rate of production it achieved around 48µg mg $^{-1}$. These data are higher than that found by O`Kennedy et al. (2000) working with shake flasks who found 9.12µg mg $^{-1}$ plasmid yields. Generally, volumetric plasmid yields achieved in batch cultivation tend to be

relatively low, with values ranging from 4 to 40mg L^{-1}. It was observed that the increase of velocity of agitation promoted the increase of the specific production of biomass. However, the increase of agitation decreased the specific production of DNA plasmidial in function of biomass for both used culture medium. When employing rich cultivation media, the fermentor oxygen transfer capacity is eventually exceeded, resulting in the creation of an oxygen-limited environment. This lack of oxygen triggers the fermentative metabolism of *E. coli*, rapidly leading to the production of toxic by-products, mostly acetic acid, that severely limit growth and even lead to cell death (Cherrington et al., 1990; Luli and Strohl, 1990). The results obtained in this work demonstrated that the conditions environmental used lead to elevated plasmid production in TB culture medium, supplemented with 30μg mL^{-1} of kanamycin under agitation of 200rpm are conditions adequate for the production of the plasmid DNA in study.

Plasmid stability

In Figure 4 the data of the plasmid stability are shown. The plasmid stability was examined during growth of recombinant *E. coli.* It is observed that TB medium provided better stability plasmid as a function time. However, for the LBG medium there was reduction in the stability. Plasmid stability can be structural, through modification of the plasmid DNA, or segregational, by the generation of plasmid-free cells during the fermentation, host strain used for transformation and environmental factors (Chaves et al., 1999). O´Kennedy et al. (2000) evaluated plasmid stability when employing several media (complex LB versus semi-defined formulations) and found that a semi-defined medium supported the highest plasmid stability, while the complex LB medium offered the lowest plasmid stability. Media that supported similar growth rates between plasmid-bearing and plasmid-free cells also supported high plasmid stability. The authors concluded that lower plasmid stability resulted from a

wider growth rate difference achieved when using certain formulation, which conferred an advantage to the plasmid-free cells. Environmental factors such as limiting nutrient (carbon, nitrogen and phosphorous) supply during fermentation can deeply affect the microbial physiology and the cellular response to plasmid stability (Lima Filho and Ledingham, 1992). The effects of medium composition on plasmid production are closely intertwined composition on plasmid segregational stability. An important factor contributing to plasmid stability is the presence of an antibiotic, selecting for cells containing the desired plasmid in the cultivation medium. Antibiotic presence in the cultivation medium is not problematic since clearance, or clearance of its inactivated form, can be readily achieved during the downstream steps (Swartz, 2001). However, the mechanisms of resistance encoded by the plasmid usually lead to either deactivation or degradation of the antibiotic. In liquid culture, selective pressure may diminish with time as deactivation of the antibiotic occurs. Therefore, a potential for plasmid loss exists due to inherent instability, even when antibiotics are added to the cultivation medium. The mechanisms of plasmid instability observed, dimmer formation, segregational instability, and degradation, probably remain valid (Prather et al., *in press*). Medium composition and cultivation conditions play an important role by controlling plasmid copy number, stability and the amount of biomass (Greasham and Herber, 1997). Chemically defined formulations offer the possibility to perform extensive analytical investigations, which in turn can support metabolic studies and quality control purposes. In addition, the use of chemically defined media eliminates most of the uncertainty facing the origin and composition of the raw complex ingredients. In the context of developing processes for the commercial production of vaccines, the use of chemically defined cultivation media will also help in achieving a better position with respect to the regulatory environment by supporting safety and reproducibility claims (Zhang and Greasham, 1999).

Conclusion

The plasmid production in shake flasks was highly dependent on the cultural conditions. The presence of different selective pressure concentrations (kanamycin) influenced the production of plasmid DNA. Concentrations among 30-50μg mL^{-1} of kanamycin demonstrated behavior in the assays of biomass and plasmid DNA production. As well as the different used agitations, with the increase of the velocity of agitation (200 rpm) it occurs increase of the biomass production however it occur decrease of plasmid DNA. TB medium presented the best results in terms of DNA plasmid production and it showed to be most stable when compared to LBG medium.

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References

Baheri, H.R., Hill, G.A., Roesler, W.J., 2001. Modelling plasmid instability in batch and continuous fermentors. Biochem. Eng. J. 8, 45-50.

Chaves, A.C., Abath, F.G.C., Lima Filho, J.L., Cabral, J.M.S., Lucena-Silva, N., 1999. Studies on growth kinetics and plasmid stability of a recombinant *Escherichia coli* expressing a *Schistosoma mansonii* antigen. Bioproc. Eng. 21, 355-361.

Cherrington, C., Hinton, M., Chopra, I., 1990. Effect of short-chain organic acids on macromolecular synthesis in *Escherichia coli.* J. Bacteriol. 68, 69-74.

Ferreira, G. N. M., Monteiro, G. A., Prazeres, D. M. F., Cabral, J. M. S., 2000. Downstream processing of plasmid DNA for gene therapy and DNA vaccine application. Tibtech 18, 380-388.

Greasham, R., Herber, W., 1997. Design and optimization of growth media. In: Applied microbial physiology – a practical approach. Oxford: Oxford University Press, p.53-74.

Gupta, R., Sharma, P., Vyas, V. V., 1995. Effect of growth environment on the stability of a recombinant shuttle plasmid, pCPPS-31, in *Escherichia coli*. J. Biotechnol. 41, 29-37.

Levy, M. S., O' Kennedy, R. D., Ayazi-Shamlou, P., Dunnil, P., 2000. Biochemical engineering approaches to the challenges of producing pure plasmid DNA. Tibtech 18, 296-305.

Lima Filho, J.L., Ledingharm, W.M., 1992. Uptaken of ammonia by *Saccharomyces cerevisiae* carrying the plasmid pCYG4 related with ammonia assimilation. Appl. Biochem. Biotechnol. 36, 107-112.

Lu, Y., Raviprakash, K., Leão, I.C., Chikhlikar, P. R., Ewing, D., Anwar, A., Chougnet, C., Murphy, G., Hayes, C. G., August, T. J., Marques, E. T., 2003. Dengue 2 PreM-E/LAMP chimera targeted to the MHC class II compartment elicits long-lasting neutralizing antibodies. Vaccine, 21, 87-92.

Luli, G., Strohl, W., 1990. Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. Appl. Environ. Microbiol., 56, 1004-1111.

O' Kennedy, R. D., Baldwin, C., Keshavarz-Moore, E., 2000. Effects of growth medium selection on plasmid DNA production and initial processing steps. J. Biotechnol. 76, 175-183.

Prather, K.J., Sagar, S., Murphy, J., Chartrain, M., 2003. Industrial scale production of plasmid DNA for vaccine and gene therapy: plasmid design, production, and purification. Enz. Microbiol. Technol. *In press*.

Rhee, J.I., Bode, J., Diaz-Ricci, J.C., Poock, D., Weigel, B., Kretzmer, G., Schugerl, K., 1997. Influence of the medium composition and plasmid combination on the growth of recombinant *Escherichia coli* JM109 and on the production of the fusion protein *Eco*RI:SPA. J. Biotechnol. 55, 69-83.

Ribeiro, S. C., Monteiro, G. A., Cabral, J. M. S., Prazeres, D. M. F., 2002. Isolation of plasmid DNA from cell lysates by aqueous two-phase systems. Biotechnol. Bioeng. 78, 376-384.

Sambrook, J., Fritch, E. F., Maniatis, T., 1989. Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Swartz, J., 2001. Advances in *Escherichia coli* production of trerapeutic proteins. Curr. Opin. Biotechnol. 12, 195-201.

Vyas, V. V., Gupta, S., Sharma, P., 1994. Stability of reombinant shuttle plasmid in *Bacillus subtilis* and *Escherichia coli*. Enzyme Microb. Tehnol. 16, 240-246.

Wang, Z., Le, G., Shi, Y., We, G., 2001. Medium design for plasmid DNA production based on stoichiometric model. Process Biochem. 36, 1085-1093.

Zhang, J., Greasham, R., 1999. Chemically defined media for commercial fermentations. Appl. Microbiol. Biotechnol. 51, 407-421.

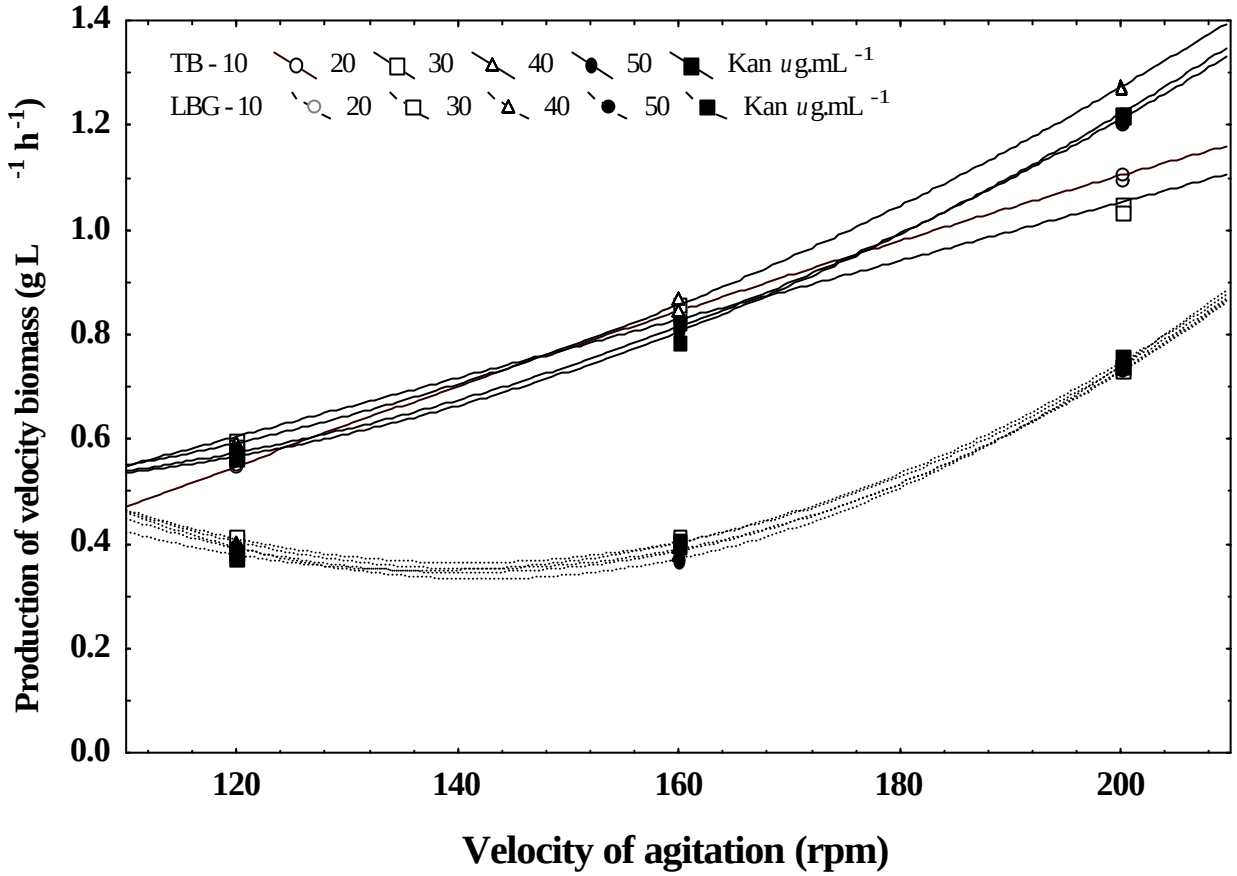
TB - 10 20 30 40 50 Kan ug.mL -1
LBG - 10 20 30 40 50 Kan ug.mL -1
Production of velocity biomass (g L -1 h-1)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
120
140
160
180
200
Velocity of agitation (rpm)

FIGURE 1

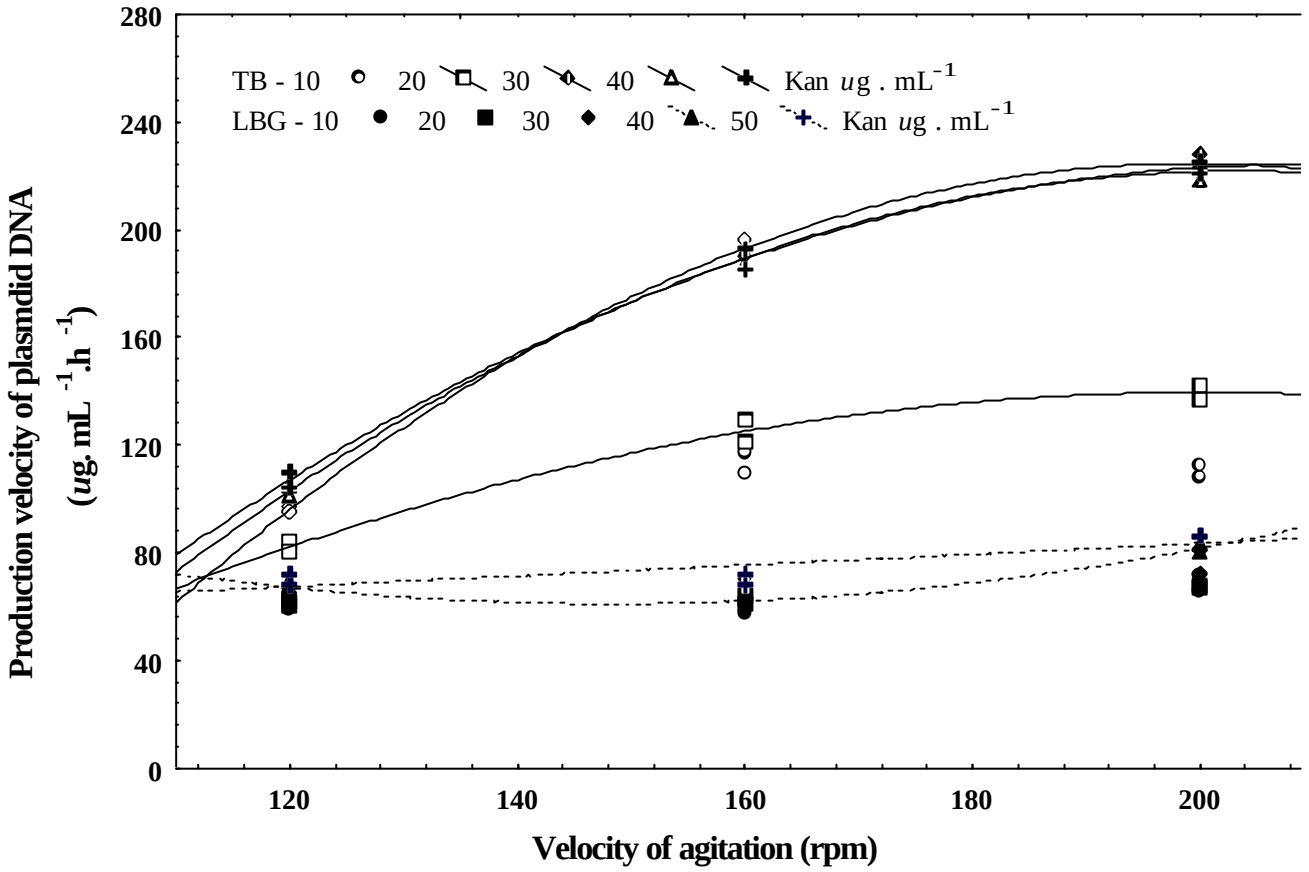
TB - 10 20 30 40 Kan ug . mL-1
LBG - 10 20 30 40 50 Kan ug . mL-1
Production velocity of plasmdid DNA
(ug. mL -1.h -1)
280
240
200
160
120
80
40
0
120
140
160
180
200
Velocity of agitation (rpm)

FIGURE 2

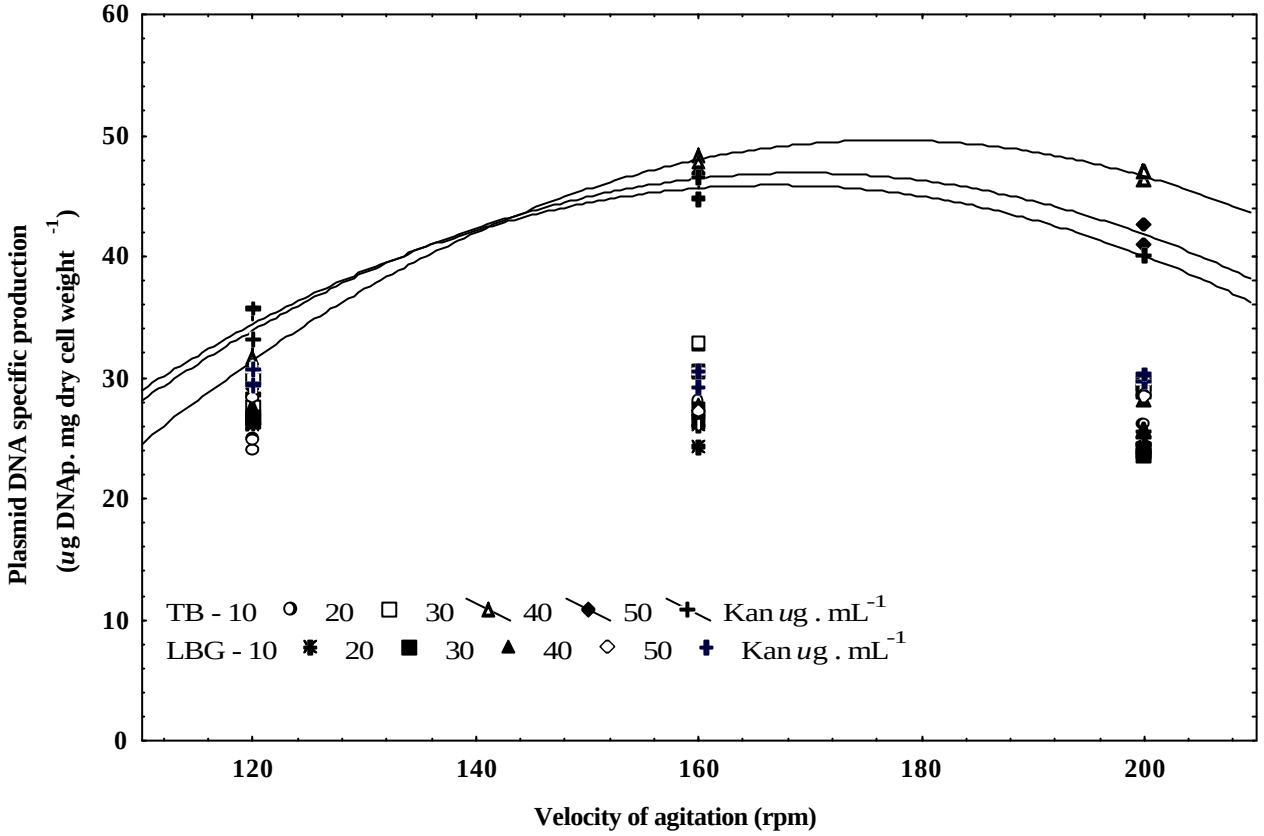
Plasmid DNA specific production
(ug DNAp. mg dry cell weight -1)
60
50
40
30
20
10
0
120
140
160
180
200
Velocity of agitation (rpm)
TB - 10
20
30
40
50
Kan ug . mL-1
LBG - 10
20
30
40
50
Kan ug . mL-1

FIGURE 3

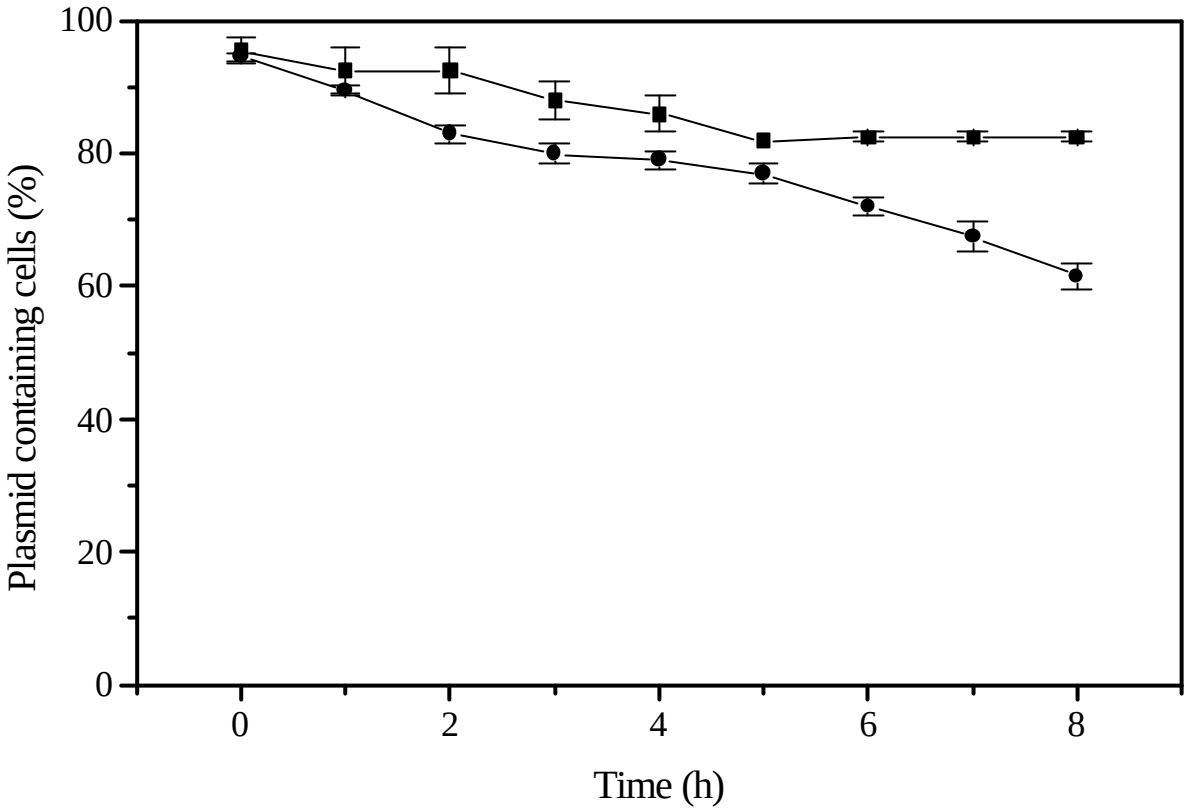

FIGURE 4

Figure caption

Figure 1. Biomass production velocity to recombinant *E. coli*: culture media, kanamaycin concentration and velocity of agitation effects. TB (—) and LBG (- - -).

Figure 2. Production velocity of plasmid DNA to recombinant *E. coli*: culture media, kanamaycin concentration and velocity of agitation effects. TB (—) and LBG (- - -).

Figure 3. Plasmid DNA specific production cultivation of recombinant *E. coli* at different media.

Figure 4. Plasmid DNA stability during cultivation of recombinant *E. coli* at different medium. Percentage of plasmid-containing cells at media TB (▲) and LBG (●).

Capítulo 2 - Extraction of dengue 2 plasmid DNA vaccine (pD2) from cell lysates by aqueous two-phase systems - **Manuscrito submetido para publicação na revista "Journal of Chromatography B"**.

Extraction of dengue 2 plasmid DNA vaccine (pD2) from cell lysates by aqueous two-phase systems

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Extraction of dengue 2 plasmid DNA vaccine (pD2) from cell lysates by aqueous two-phase systems

Abstract

This work describes the partitioning in PEG/phosphate systems of the plasmid pD2, a dengue 2 plasmid DNA vaccine, present in a clarified *E. coli* alkaline lysate. Factors that affect the partition as PEG molecular weight, plasmid concentration, and the lysate volume loaded in the system were investigated. Results showed that partition behaviour of plasmid DNA depends on the system molecular weight, while a good amount of protein of the cell lysate was accumulated in the interphase of the systems. The best recovery plasmid yield (37%) was obtained with PEG 400 (20/20% w/w) systems with a 60% (w/w) lysate load.

Keywords: Aqueous two-phase systems, liquid-liquid extraction, plasmid DNA

1. Introduction

The widespread interest in gene therapy and DNA-based vaccination has led to an increased demand for large amount of pure plasmid DNA [1-3]. It is urgent to develop new methods to purify plasmids with high yields and minimal or no contamination [4]. The cost of the recovery of products with importance for pharmaceutical/clinical research for industrial use becomes critical to the overall process economics, representing 50-90% of the total cost [5, 6]. Aqueous two-phase systems (ATPS) had been widely and successfully used on the extraction and purification of biological macromolecules [7,8]. Over 40 years ago, Albertsson [9] was one of the first to study the separation of biomolecules and particles in ATPS in a systematic way. The technique later proved to be of immense utility in analytical, biochemical and environmental research and applications [10]. As opposed to proteins, plasmid purification by aqueous two-phase extraction has evolved very little and few related have appeared in the literature in the last years [11-13]. Aqueous two-phase systems are formed by mixing two polymers or a polymer and salt above some threshold concentration. Both phases contain a high proportion of water (80-95%) providing a nontoxic environment for biomolecules and low interfacial tension, they provide mild conditions especially suited for biological macromolecules separation [14-16]. Separation is achieved by the different distribution, between the two phases, of the target compound and the contaminants. The mechanism of partition is not well understood and separation of compounds is usually attained by a systematic variation of system composition [17, 10]. This includes type, molecular weight and concentration of polymer, type and concentration of salt and pH. The partition of nucleic acids in ATPS depends on many factors, such as the size and chemical properties of the macromolecule, the properties of the system components, and the ionic composition [18-21]. A change in the systems properties will change the surface properties of the

partitioning solutes and thus affects partitioning. Some of the interactions between solutes and phase components must involve hydrogen bonds, charge interaction, van der Waals forces, hydrophobic interaction and steric effects [14]. Overall, the prediction partitioning is a difficult task, particularly in the case of large molecules [11]. Here we describe the partitioning of an plasmid pD2, a dengue 2 plasmid DNA vaccine [22], present in a clarified *E. coli* alkaline lysate, in PEG/phosphate systems. Factors that affect the partition, PEG molecular weight, plasmid concentration, and the lysate volume loaded the system were investigated.

2. Experimental

2.1 Materials

Polyethylene glycol (PEG) 300, 400, 550, 1000 and 8000 were purchased from Sigma Chemical Company (St Louis, MO, USA). Di-potassium hydrogen phosphate was from Nuclear (São Paulo, Brazil). Pico Green® ds DNA quantization reagent was acquired from Molecular Probes (Leiden, The Netherlands). In all experiments were applied the Plasmid pD2 a dengue 2 plasmid DNA vaccine, expressing the virus pre-membrane and envelope proteins [22]. All the other reagents used were of analytical grade.

2.2 Plasmid and bacterial strain

The plasmid was transformed and propagated in *Escherichia coli* XL1 Blue. Recombinant bacteria were stored in 25% (v/v) glycerol at -80ºC [23].

2.3 Production of bacterial culture

Bacteria were grown overnight in 1000 ml shake flasks containing 250 ml of Terrific Broth medium (20 g tryptone L^{-1}, 24 g yeast extract L^{-1}, 4 ml glycerol L^{-1},

0.017 M KH_2PO_4, 0.072M K_2HPO_4) supplemented with 30µg/ml of kanamycin, at 37ºC and 160 rpm. *E. coli* XL1 Blue cells without plasmid were grown in similar conditions but without antibiotic.

2.4 Cell lysis

A modified alkaline method was used for cell lysis [23]. Cells (250 ml) were harvested by centrifugation at 15,000g (20 min, 4ºC) the pellets were resuspended in 12.5 ml of 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0. The cells were lysed by adding gently (10 min on ice) 12.5ml of 200 mM NaOH 1% (w/v) SDS during 10 min on ice. The lysate was neutralized with 9.4 ml of a solution of 3 M potassium acetate, 11.5% (v/v) glacial acetic acid (10 min on ice). All the solutions were previously chilled. The precipitate was removed by centrifugation (15,000g, 30 min, 4ºC) and the lysate was kept at –20ºC for further plasmid DNA recovery and purification with ATPS.

2.5 Plasmid standards

Plasmid standards were prepared from *E. coli* cultures using the Flexi Prep kit, Amershan Pharmacia, resuspended in TE buffer (10 mM Tris/HCl, 1mM EDTA, pH 8.0) and quantified by measuring the absorbance at 260 nm.

2.6 Aqueous two-phase systems

The PEG/K_2HPO_4 ATPS were prepared with typical concentrations for the chosen PEG molecular weights: PEG 300 (20/20% w/w), 400 (20/20% w/w), 550 (20/20% w/w), 1000 (15/13% w/w) and 8000 (10/10% w/w). The extraction mixtures, with a total mass of 5 g, were prepared in 15 ml conical tubes. The amount of neutralized lysate loaded was 1, 2 or 3 g (corresponding to 20, 40 or 60% w/w). After

the addition of all the components of the systems, it was agitated in vortex. This mixture was then centrifuged for 1 min at 1000 g to facilitate phase separation. Top and bottom phases were carefully isolated and stored at 4ºC for further analysis. Each phase (15 µl) was analyzed by electrophoresis in 1% agarose gels run with TAE buffer in the presence of 0.5 µg ml^{-1} ethidium bromide. The controls of the aqueous two phase systems of phases had been made with the same composition of the ones described above using the *E. coli* lysate without plasmid (XL1 Blue lysate). The procedures followed to obtain top and bottom phases were the same.

2.7 Plasmid DNA analyses

Plasmid DNA was quantified by fluorescence analysis using Pico Green® (Molecular Probes, Inc, USA) an ultra-sensitive fluorescent stain that binds specifically to double-stranded nucleic acids. Before each set of measurements, a Pico Green® stock solution as per manufacture's instructions was prepared. The fluorescence was measured in 490 nm excitation and 520 nm emission, using a spectrometer (Yvon Jobin, France) connected to a laser (Innova/Coherent, USA). In order to quantify the plasmid in the pD2 lysate, a calibration curve was made using the lysate without plasmid (XL1 Blue lysate). The XL1 Blue lysate was diluted using sterile TE buffer. Calibration standards (5-60 ng ml^{-1}) were prepared by adding known amounts of pure pD2 plasmid to this diluted XL1 Blue lysate. For the quantitatation of plasmid in the ATPS blanks, the blanks top and bottom phases were diluted with sterile TE buffer. Calibration standards (5-60 ng ml^{-1}) were prepared by adding known amounts of pure pD2 plasmid to these diluted top and bottom phases [1].

2.8 Protein analysis

The protein ATPS was estimated using a modification bicinchoninic acid (BCA)

assay (Micro well plate protocol; Pierce, Rockford, IL, USA). To overcome the interference of PEG and salt in the samples, a series of calibration curves were constructed with appropriate ATPS blanks prepared as follows. A mixture of the buffers used in the preparation of the lysates (here after named mixture X) with exactly the same final composition was made. The blanks were then prepared by replacing the lysate in the ATPS preparation with the mixture X. Top and bottom phases were carefully separated and kept at 4ºC. Calibration curves were then carried out by adding bovine serum albumin (concentrations up to 250 μg/ml) to each top and bottom phases of the previously prepared ATPS blanks. The calibration curve used for the quantization of protein in the lysate was made by adding BSA directly to the mixture X. For analysis, 100 μl of each sample were mixed with 100 μl of sodium deoxycholate (0.15% w/v) with 800 μl sterile distilled water. After 10 min at room temperature, 100 μl of trichloroacetic acid (72% w/v) was added. Samples were then vortex mixed and centrifuged for 20 min at 8,000g. The supernatant was removed and pellets solubilized in 50μl of sodium dodecyl sulphate (5% w/v) containing 0.1M NaOH, then BCA reagent was added (200 μl) and the samples were incubated at 60ºC for 30 min [11]. Absorbance was measured at 595 nm in Bio-Rad (Hercules, CA) model 550 micro plate reader.

3. Results and discussion

3.1 Plasmid and RNA Partitioning

In systems of aqueous two-phase composed by PEG 300, 400 and 550 for concentrations of 20 and 40% (w/w) of lysate a well-defined white interphase was observed. For 60% (w/w) concentration a white interphase was also observed in systems with PEG 300, 400, 550 and 1000. Agarose gel analysis of the interphase material (data not shown) corfirmed that, in all systems, plasmid and RNA were lost to in the

interphase area. Studies carried out by Kimura [24] with potassium phosphate-PEG aqueous two-phase system (PEG 1500 and 3000) in the RNA partition, showed that the RNA of low-molecular-mass was partitioned between the top and bottom phases, if partitioned alone. However, the RNA low-molecular-mass was caught in the interphase to a significant extent, if partitioned with the coexisting RNA high-molecular-mass. In the current work, the studied systems, the RNA was partitioned towards the systems where the plasmid was partitioned (Fig. 1A, B and C). Probably the RNA that constitutes cell lysate has low-molecular-mass, therefore partitioned between the two phases (top and bottom), depending on the PEG molecular weight of the system. By the analysis in the gel of agarose it was observed that the plasmid was partitioned in the PEG rich higher phase for PEG 300 and 400, while for PEG 1000 and PEG 8000 systems the plasmid was partitioned in the salt rich phase (Figure 1 A, B and C). However, using PEG 400 (60% w/w) system probably the plasmid was to the interphase area. In these figures the plasmid shows two conformations in the cell lysate solution in the ATPS, one corresponding to the plasmid in its open circular (oc) and the other in a supercoilded (sc) form. Probably these forms are due to the proper cellular process of lyse, by the presence of high concentrations of salt and others ion presents in the solution or as result of the plasmid instability [1]. The partitioning of plasmid in ATPS is complex and influenced by a large number of factors [11]. In PEG-salt systems, one of the major factors is the interaction that exists between the components (other than the solute) in each phase. In these systems the energy of each phase that arises from these 1interactions is considerably different. Top phases are dominated by the repulsive interaction between PEG and salt, and bottom phases by the strong attraction of salt to water. Accordingly, solutes (plasmid and RNA molecules, in this work) will prefer the top phase that has PEG with low molecular weight, since disrupting interactions between its components is energetically favorable [25]. The type, salts

concentration, and the ratio between different ions in both phases, is particularly important for highly charged molecules such as nucleic acids [14]. The partition of a charged solute is influenced by the unequal distribution of ions due to different affinites for the phases, which generates an electrical potential between the phases, $\Delta\psi$, defined as ψ_{top} - ψ_{bottom}. The magnitude and sign of $\Delta\psi$ are determined by the partitioning behavior of ions from the majority abundant salt in the system. In PEG-phosphate systems, the phase-forming salt will, therefore, determine $\Delta\psi$. In these systems $\Delta\psi$ is positive, for the PEG-phosphate and PEG-sulphate systems, so that it favors partitioning of net negatively charged biomolecules into the PEG-rich top phase [25]. The phenomena of the collapse of DNA macromolecules in aqueous solutions of PEG can also play a role in the partitioning of plasmid to the salt-rich phase. At low PEG molecular weight (or low PEG concentrations) the flexible polymer chains can penetrate inside of the DNA which adopts a swollen coil conformation, and regime of good compatibility between PEG and DNA. If the PEG molecular weight is higher (or if more PEG is added) the solvent quality for DNA becomes poorer and the effective attraction between DNA segments in the macrolecules increases. At a certain point, a discrete transition occurs when the DNA coil contracts abruptly to form a compact globular structure. In this regime of perfect incompatibility, there is segreagation between DNA chains and PEG molecules [26].

3.2 Protein Partitioning

The results of the protein partitioning are shown in the Fig. 2A, 2B and 2C. In systems with 20% (w/w) of lysate (Fig. 2A) it was observed a great accumulation around 90-76% of protein (PEG 300 to 1000) in the interphase. While for systems with PEG 8000 the partitioned protein was around 60%. It was also observed that in all the studied systems and all for the concentrations of cell lysate occur partition of the

protein for both the phases, top and bottom, except in PEG 300 system (20% (w/w) of cell lysate) it did not occur partition for the low phase. With the increase of the loaded volume of cell lysate occurs reduction of the accumulation of protein in the interphase. These results are in accordance with published studied, which revealed that the majoriaty of intracellular proteins show changes in preference phase when it was partitioned in PEG-phosphate systems with PEG molecular weight between 1000 and 2000 [11]. The preference for the top phase seen for pH values above the proteins isoeletric point is lost when higher PEG molecular weights are used due to excluded volume effects [27]. Shibusawa et al. [28] using aqueous two-phase systems PEG 1000 (16%)/potassium phosphate (12.5%) carry on purification of single-strand DNA binding protein from an *Escherichia coli* lysate demonstrated that purification occurs using this solvent system, which has conventionally been used to separate several proteins. We select for this work systems formed by PEG 300, 400 and 1000 the lives promising ones will be plasmid isolation and therefore, will be selected to further partitioning studies.

3.3 Effect of lysate load

The effect of lysate load (20, 40 and 60% of total system mass) on plasmid extraction yield was analyzed for each of the selected ATPS. The plasmid in the cell lysate, and in the top and bottom phases obtained after extraction was quantified by fluorescence analysis. Calibration curves were constructed for all the systems tested. The slopes were obtained from the corresponding linear regressions. The interception values constitute an indication of the amount of impurities (gDNA, RNA and proteins) present in the phase, which increase the fluorescence signal. In all systems this value increased with an increase in the lysate load. These observations are in accordance with the agarose gel analysis (Fig 1A, B and C) that shows a significant increase in RNA

concentration with the lysate load for the bottom phase of systems PEG 1000. Ribeiro et al. [11] working with plasmid pCF1-CFTR isolated in PEG/salts phosphate systems obtained similar results to the presented ones in the present work. The amount of lysate loaded to the systems (20, 40 or 60%) also affects the partitioning (Table 1). In this table the results of the recovery plasmid and the protein are presented. It is observed that with increased of the loaded volume in the systems it is increased recovery of the plasmid and the protein. The loss of plasmid to the interphase is responsible for the low recovery yields obtained in some of the systems. The addition of increasing volumes of cell debris and components, changes the position of the binodal curve in phase diagrams by displacing it towards the origin [29,30]. This means that for each PEG molecular weight, the difference in composition of the top an bottom phases increases with the lysate load, providing an increased driving force for the unequal partition of the solute [26]. This was observed for plasmid partitioning with all the used systems where the plasmid recovery yield increased with the lysate load.

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References

[1] S.C. Ribeiro, G.A. Monteiro, G. Martinho, J.M.S. Cabral, D.M.F. Prazeres, Biotechnol. Letters 22 (2000) 1101.

[2] A. Mountain, Tibtech 18 (2000) 119.

[3] I.S. Noites, R.D.O'Kennedy, M.S. Levy, M. Abidi, E. Keshavarz-Moore, Biotechnol. Bioeng. 66 (1999) 195.

[4] G.N.M., Ferreira, G.A. Monteiro, D.M.F. Prazeres, J.M.S. Tibtech. 18 (2000) 380.

[5] M.J. Costa, M.T. Cunha, J.M.S. Cabral, M.R. Aires-Barros, Bioseparation, 9 (2000) 231.

[6] K.S.M.S., Raghavarao, N.K. Rastogi, M.K. Gowthaman, N.G. Karanth, Adv. Appl. Microbiol. 41 (1995) 97.

[7] J.C. Marcos, L.P. Fonseca, M.T. Ramalho, J. Chromatogr. B 711 (1998) 295.

[8] M. Pereira, Y.-T. Wu, A. Venâncio, Biochem. Eng. J. 15 (2003) 131.

[9] P-A, Albertsson, Arch. Biochem. Biophys. 1 (1962) 264.

[10] R. Hatti-Kaul, Methods in Biotechnology, v.11, Humana Press, New Jersey, 1999, pp. 1-10.

[11] S.C. Ribeiro, G.A. Monteiro, J.M.S. Cabral, D.M.F. Prazeres, Biotechnol. Bioeng. 78 (2002) 376.

[12] K.D. Cole, Biotechniques 11 (1991) 18.

[13] R. Ohlsson, C.C. Hentschel, J.G. Williams, Nucl. Acids. Res. 5 (1978) 583-590.

[14] P.A. Albertsson, Partition of Cell Particles and Macromolecules, Jonh Wiley, New York, 1986, 346p.

[15] J.M.S. Cabral, M.R. Aires-Barros, Recovery Process for Biological Materilas, John Wiley, New York, 1993, pp. 273-301.

[16] M.R. Kula, H. Kroner, H. Hustedt, Biochem. Eng. 24 (1982) 73.

[17] J.C. Marcos, L.P. Fonseca, M.T. Ramalho, J.M.S. Cabral, J. Chromatogr. B 734 (1999) 15.

[18] P.A. Albetsson, Biochim. Biophys. Acta 103 (1965) 1.

[19] G. Frick, T, Lif, Arch. Biochem. Biophys. 1 (1962) 271.

[20] T. Lif, G. Frick, P-A. Albertsson, J. Mol. Biol. 3 (1961) 727.

[21] W. Miller, in H. Walter, D.E. Brooks, D. Fisher (Editors) Partioning of Nucleic Acids, Academic Press, Orlando, FL, 1985, pp. 227-266.

[22] Y. Lu, K. Raviprakash, I.C. Leão, P. R. Chikhlikar, D. Ewing, A. Anwar, C. Chougnet, G. Murphy, C. G. Hayes, T. J. August, E. T. Marques, Vaccine 21 (2003) 2187.

[23] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning – A Laboratory Manual, Cold Spring Harbour Laboratoy Press, New York, 1989.

[24] K. Kimura, J. Chromatogr. B 734 (2000) 421.

[25] H-O. Johansson, G. Karlstron, F. Tjerneld, C.A. Haynes, J. Chromatogr. B 711 (1998) 3.

[26] V.V. Vasilevskaya, A.R. Khokhlov, Y. Matsuzawa, K. Yoshikawa, J. Chem. Phys. 102 (1995) 6595.

[27] J. Huddleston, K.W. Ottomar, D. Ngonyani, J.A. Flanagan A. Lyddiat, in D.L. Pyle (Editor) Separations for Biotechnology 2, Elsevier Applied Science, London, 1990, pp.181-190.

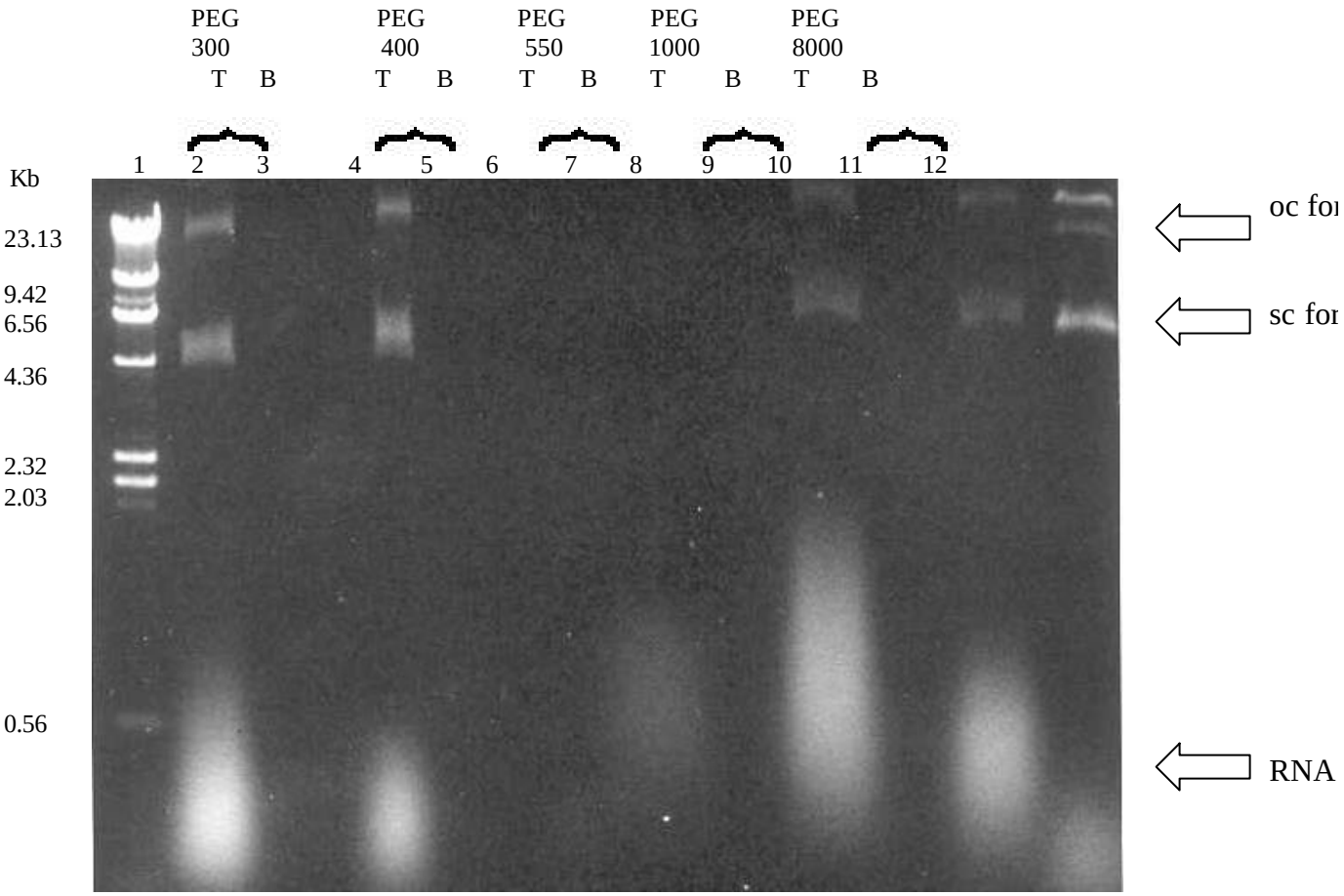
[28] Y. Shibusawa, Y. Ino, T. Kinebuchi, M. Shimizu, H. Shindo, Y. Ito, J. Chromatogr. B *in press.*

[29] K.K. Alstine, A. Veide, in R. Hatti-Kaul (Editor), Methods in Biotechnology, vol. 11, Humana Press, New Jersey, 1999, pp.251-258.

[30] J. Huddleston, A. Veide, K. Kohler, J. Flanagan, S-O. Enfors, A. Lyddiat, Trends Biotechnol. 9 (1991) 381.

Table 1. Concentrations of plasmid and protein in the lysate and after extraction with ATPS.

System	Lysate load % (w/w)	Plasmid (µg/ml)	Recovery plasmid yield (%)	Protein (µg/ml)	Recovery protein yield (%)
Lysate	-	238.0	100.00	986.00	100.00
PEG 300	20	52.0	21.84	78.88	8.00
Top phase	40	53.0	22.26	411.16	41.70
	60	67.0	28.15	354.96	36.00
PEG 400	20	82.0	34.45	177.48	18.00
Top phase	40	83.0	34.87	364.82	37.00
	60	88.5	37.18	640.90	65.00
PEG 1000	20	25.0	10.50	39.44	4.00
Bottom phase	40	26.0	10.92	78.88	8.00
	60	32.0	13.44	78.88	8.00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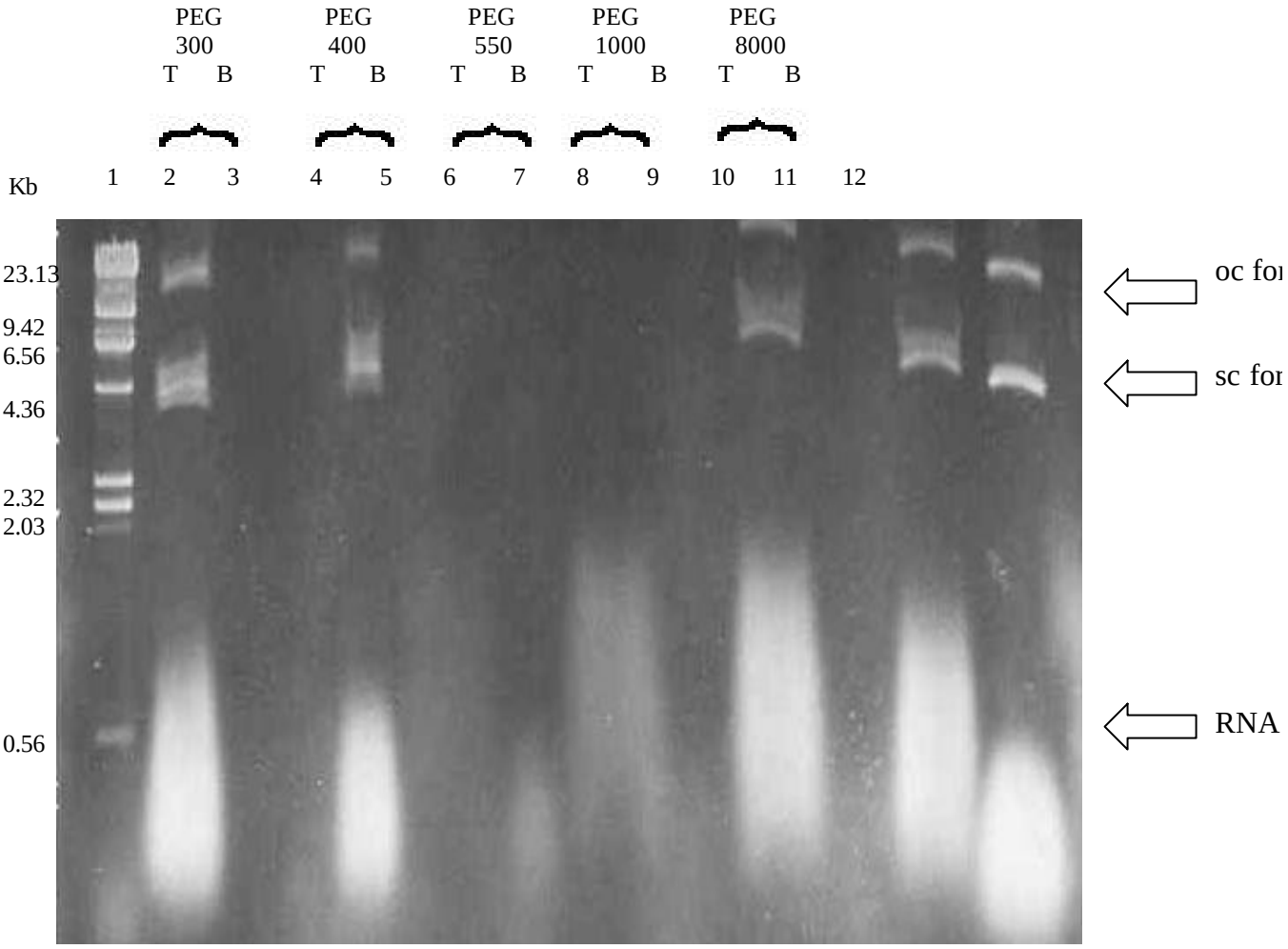
PEG 400
PEG 550
PEG 1000
PEG 8000
T B
T B
T B
T B
T B
1 2 3 4 5 6 7 8 9 10 11 12
Kb
23.13
9.42
6.56
4.36
2.32
2.03
0.56
oc for
sc for
RNA

Figure 1A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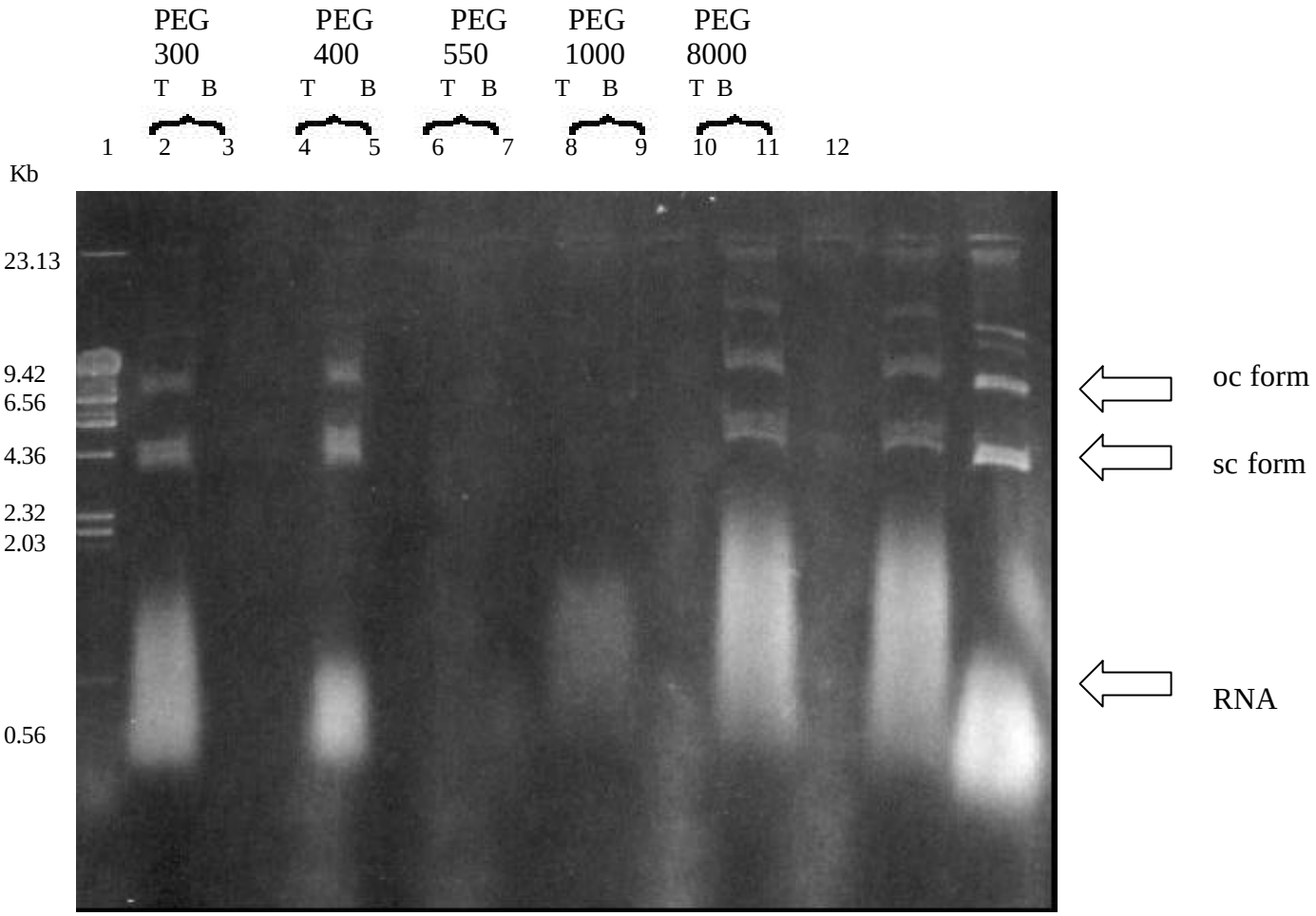
T B
PEG 400
T B
PEG 550
T B
PEG 1000
T B
PEG 8000
T B
Kb
1 2 3 4 5 6 7 8 9 10 11 12
23.13
9.42
6.56
4.36
2.32
2.03
0.56
oc for
sc for
RNA

Figure 1B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PEG 400
PEG 550
PEG 1000
PEG 8000
T B
T B
T B
T B
T B
1 2 3 4 5 6 7 8 9 10 11 12
Kb
23.13
9.42
6.56
4.36
2.32
2.03
0.56
oc form
sc form
RNA

Figure 1C

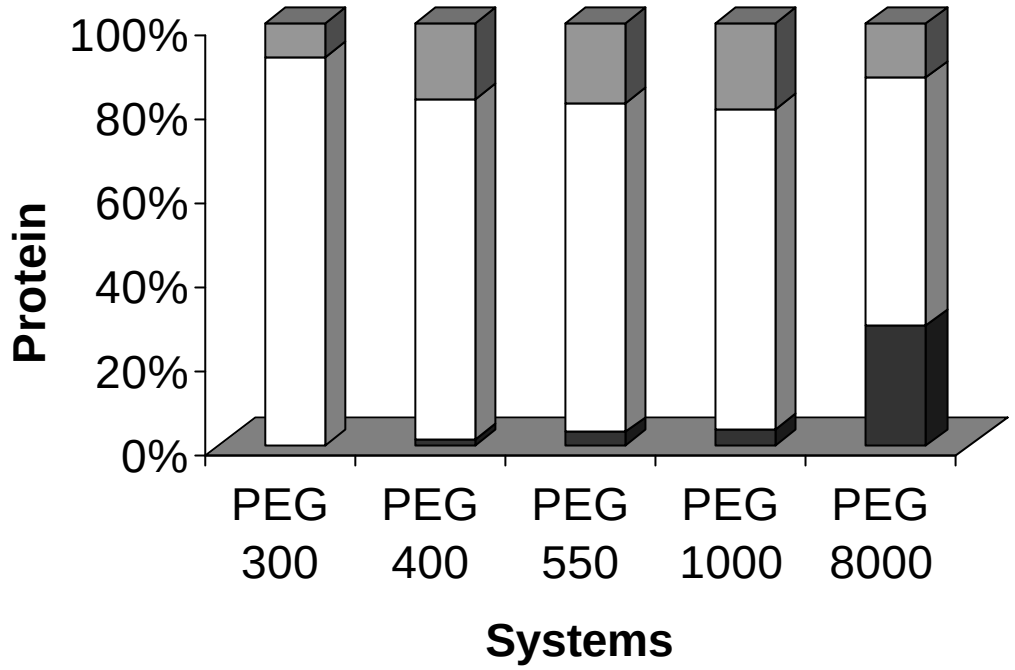

100%
80%
60%
40%
20%
0%
Protein
PEG 300
PEG 400
PEG 550
PEG 1000
PEG 8000
Systems

Figure 2A

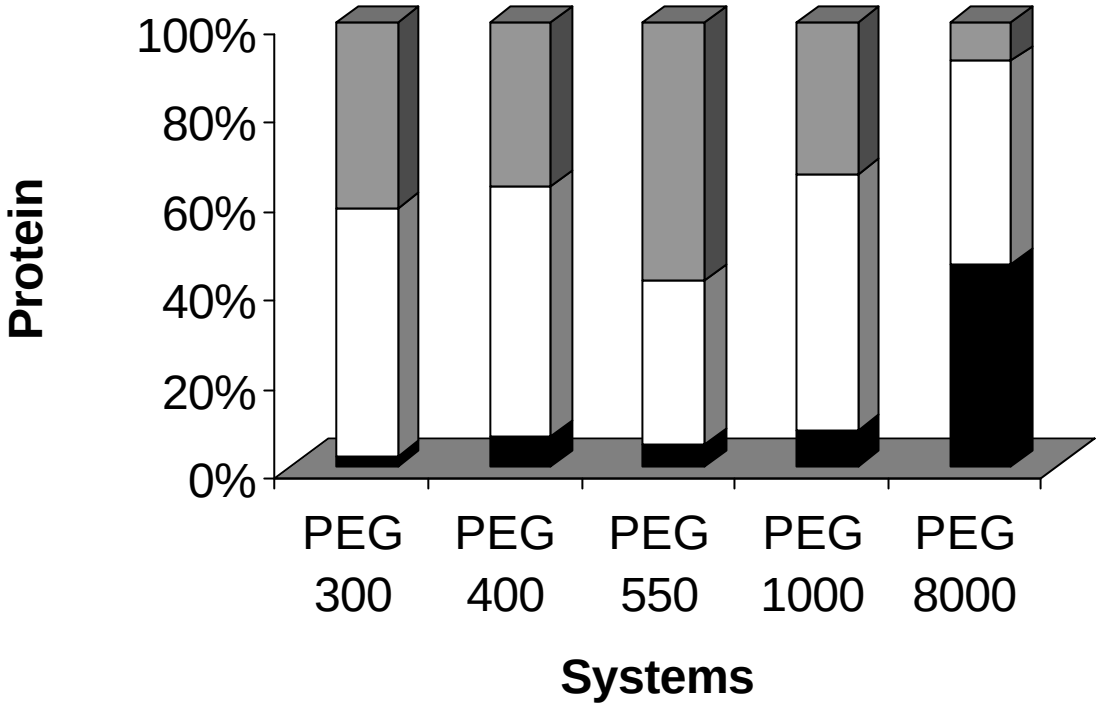
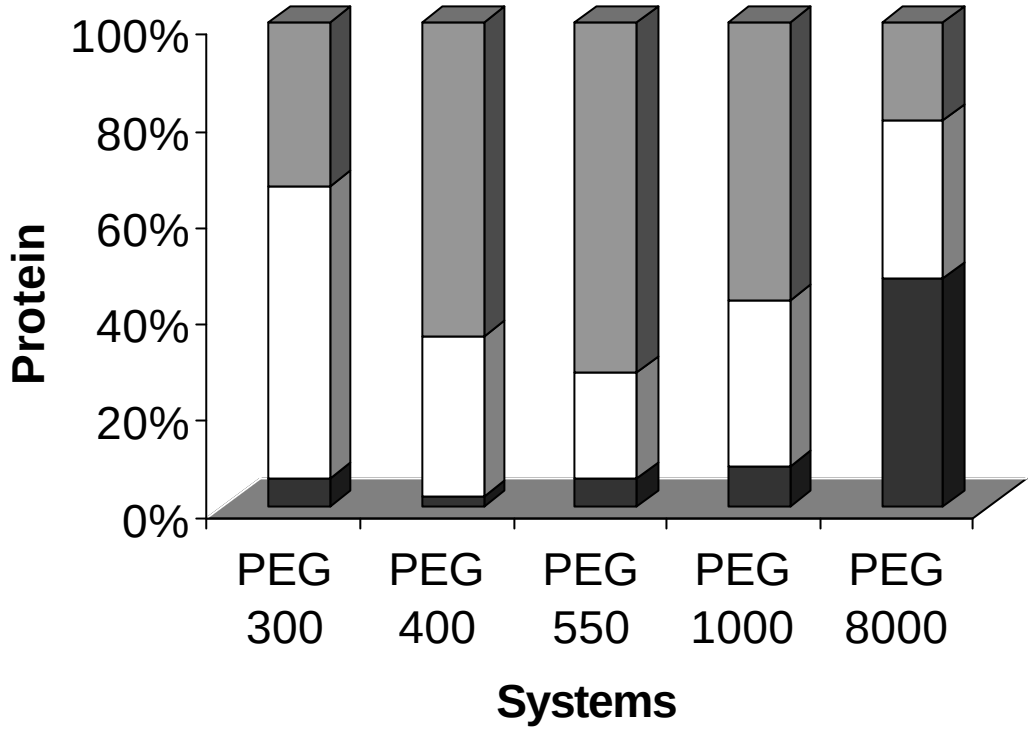

Protein
100%
80%
60%
40%
20%
0%
PEG 300
PEG 400
PEG 550
PEG 1000
PEG 8000
Systems

Figure 2B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20%
0%
Protein
PEG 300
PEG 400
PEG 550
PEG 1000
PEG 8000
Systems

Figure 2C

Figure caption

Figure 1A. Agarose gel analysis of plasmid and RNA partitioning in ATPS with 20% (w/w) of lysate. T-top phase. B-bottom phase. Lane1.1µg/ml of Lambda DNA/*Hind* III marker, lanes 2 to 11: 15µl of each of the indicated phase, lane 12: 15µl of the lysate. OC: open circular. SC: supercoiled.

Figure 1B. Agarose gel analysis of plasmid and RNA partitioning in ATPS with 40% (w/w) of lysate. T-top phase. B-bottom phase. Lane1.1µg/ml of Lambda DNA/*Hind* III marker, lanes 2 to 11: 15µl of each of the indicated phase, lane 12: 15µl of the lysate.

Figure 1C. Agarose gel analysis of plasmid and RNA partitioning in ATPS with 60% (w/w) of lysate. T-top phase. B-bottom phase. Lane1.1µg/ml of Lambda DNA/*Hind* III marker, lanes 2 to 11: 15µl of each of the indicated phase, lane 12: 15µl of the lysate.

Figure 2A. Protein partitioning in PEG phosphate ATPS using 20% (w/w) of lysate. The percentage of protein in the top phase (gray bars), interphase (white bars), and bottom phase (black bars) is shown for the different PEG MW.

Figure 2B. Protein partitioning in PEG phosphate ATPS using 40% (w/w) of lysate. The percentage of protein in the top phase (gray bars), interphase (white bars), and bottom phase (black bars) is shown for the different PEG MW.

Figure 2C. Protein partitioning in PEG phosphate ATPS using 60% (w/w) of lysate. The percentage of protein in the top phase (gray bars), interphase (white bars), and bottom phase (black bars) is shown for the different PEG MW.

CAPÍTULO 3 - Purification of plasmid (pVaxLacZ) by hydrophobic interaction chromatography - Manuscrito a ser submetido para a publicação na revista “Biotechnology Letters”.

Purification of plasmid (pVaxLacZ) by hydrophobic interaction chromatography

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Key words: gene therapy, hydrophobic interaction chromatography, plasmid, purification.

Abstract

Plasmid DNA used for acid nucleic vaccination or nonviral therapeutic gene transfer has to be highly purified with minimal or zero contamination. A method is described for the purification of plasmid DNA, which includes an ammonium sulphate precipitation followed by hydrophobic interaction chromatography (HIC) using Phenyl Sepharose 6 Fast Flow (low sub). The use of HIC took advantage of the more hydrophobic character of single stranded nucleic acid impurities when compared with double-stranded plasmid DNA.

Introduction

The use of the recombinant DNA with progress of the gene therapy or nucleic acid vaccination DNA has an important hole in the prevention and in the cure of diseases such as cancer and AIDS, have been increasing the need the obtaining of DNA pure plasmid (Diogo *et al.* 2000). In some research applications it is possible to use crude cell extracts of varying degrees of purity. However, plasmid DNA used for non-viral therapeutic gene transfer or nucleic acid vaccination has to be highly purified and free of contaminating components such as bacterial proteins, toxins, genomic DNA (gDNA) or RNA (Prazeres *et al.* 1999). It is now clear that the demand for large amounts of plasmid DNA will be enormous in view of the potential number of users and the prospect of applying DNA vaccines to veterinary diseases (Little-van den Hurk *et al.* 2000). Additional, the fact that product recovery costs become critical in the overall economics of modern biotechnology processes, and the need to have a process complying with the guidelines issued by regulatory agencies has increases the interest in developing methods for the downstream processing of plasmids (Ribeiro *et al.* 2002). A process for the production of plasmid DNA generally follows the steps of fermentation, primary isolations, and purification (Prazeres *et al.* 1999). Large-scale purifications require scalable methods such as column chromatography (Prazeres *et al.* 1998). The hydrophobic interaction technique used is very in the purification of therapeutic proteins, but so far there are no reports of the application of HIC to plasmid purification (Diogo *et al.* 2000). The use of HIC will take advantage of the different hydrophobic character of double-stranded plasmid DNA and other nucleic acids impurities with high content in single strands, such as RNA and denatured gDNA. The aim of this work is to study the possibity of purifying plasmid DNA using HIC as the final operation in a simple purification process.

Materials and methods

Materials

Phenyl Sepharose 6 Fast Flow (low sub) was obtained from Pharmacia (Uppsala, Sweden). RNase-DNase free was from Boehringer (Mannheim, Germany), Luria broth (LB) was from Sigma (St. Loius, MO), and agarose was from FMC (Rockland, ME). All salts used were of analytical grade.

Bacterial culture

Escherichia coli DH5α harboring pVax-LacZ (Invitrogen) was grown overnight at 37ºC, in 100 ml shake flasks containing 25 ml of LB medium with 30µg/ml kanamycin, at 200 rpm. Larger culture volumes (250 ml) were inoculated with the appropriate amount of overnight culture and incubated under the same conditions. *E. coli* DH5α without plasmid was also grown under the same conditions as described before, but with no kanamycin present.

Lysis and primary isolation

A modified alkaline method was used for cell lysis (Sambrook *et al.* 1989). After the alkaline lysis method the plasmid in the supernatant was precipitated after addition of 0.6 volumes of isopropanol during 45 min incubation at -20ºC. The plasmid was separated by centrifugation at 10 000 g during 20 min. The pellets were then redissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Next, solid ammonium sulphate was dissolved in the plasmid solution at a concentration interval between 2.0-3.0 M, followed by 15 min incubation on ice. Precipitated proteins were removed by centrifugation at 10 000 g during 20 min at 4ºC. The supernatant was then diluted and loaded directly on the HIC column. Total plasmid was quantified by anion-exchange high-performance liquid chromatography (HPLC) throughout the different steps. The

same isolation procedure was applied to DH5α cells without plasmids to check the behaviour of cell impurities in the HIC column.

Preparative chromatography

Chromatography was performed in a Pharmacia fast protein liquid chromatography (FPLC) system (P 500 pump, LCC 500 chromatography controller). An XK16/20 (20cm x 1.6cm) column (Pharmacia) was packed with 28 ml of the HIC gel. Partially purified pVax-LacZ plasmid from the ammonium sulphate precipitation was loaded onto the column and isocratic elution was carried out with 1.5 M ammonium sulphate in 10mM Tris-HCl (pH 8.0) at a flow rate of 1 ml/mim. One-milliliter fractions were collected. The absorbance of the eluate was measured continuously at 254 nm. The samples analysed by 0.8% agarose gel electrophoresis, stained with ethidium bromide (0.5 $\mu g\ ml^{-1}$).

Plasmid standards

Plasmid standards were prepared using a plasmid mini-prep kit (Qiagen, Germany) according to the instructions of the manufacturer.

Analytical chromatography

A 4.6 x 10cm polyether ether ketone (PEEK) column packed with Poros 20 PI strong anion-exchange media from Perseptive Biosystems was connected to a Merck-Hitachi HPLC system and equilibrated with 0.7M NaCl in TE buffer. Samples (100 µl) were injected and eluted at 2 ml/min. These samples were incubated with RNase-DNase free for 60 min at 37ºC before HPLC analysis. Plasmid was quantified through a calibration curve, which was constructed using pVax-LacZ standards (2-40 $\mu g\ ml^{-1}$).

Protein analysis

The protein concentration was measured by the modification micro bicinchoninic acid (BCA) assay from Pierce (Rockford, Il, USA) (Rhoderick *et al.* 1989). A calibration curve was made with bovine serum albumin (BSA) standards. An absorbance was measured at 595 nm in a micro plate reader.

Genomic DNA analysis

Genomic DNA (gDNA) was analysed by PCR. A 361 bp sequence of the ribosomal RNA gene from *E. coli* DH5α was chosen for amplification in the PCR analysis (forward primer 5´-ACA CGG TCC AGA CTC CTA CG-3´; reverse primer 5´-ACA ACC TGG AAT TCT ACC CC-3´. The volumes of the samples to analyse were 0.35µl and the final volume of the mixture of used PCR was of 50 µl (Taq DNA 1x buffer; 200 µM dNTP´s mixture, 1.5 µM of $MgCl_2$ and 2.5 U Taq polymerase, all from Promega (Madison, WI) and 0.75 µM of each primer from Interactiva (Ulm, Germany). For each sample analysed, a calibration curve was made using 0.01-100 ng de standard gDNA. PCR was performed with a first denaturation step at 94ºC for 3 min followed by 40 cycles of amplification (60ºC, 1 min; 74ºC, 1.5 min; 94ºC, 1 min) and a final extension step (60ºC, 1 min; 74ºC, 10 min). Amplified samples (20 µl) were visualized by 2% agarose gel analysis stained with ethidium bromide (0.5 µg ml^{-1}).

Results and discussion

The results of purification of the plasmid DNA are summarized in Table 1. It was observed a decrease of the plasmid mass once along the purification process. In the stage of alkaline lysis there were 8716 µg after HIC which decreased to it 1230 µg. There was an increase of purity 5.7 (lysis solution) and after HIC it was in 100%, in all

the concentrations ammonium sulphate used. The best purification factor was obtained after HIC, using precipitation with 2.5M of ammonium sulphate, which yield 51%. Studies accomplished by Diogo et al. (2000) working in the purification of plasmid using hydrophobic interaction chromatography showed similar results. Figure 1 shows the analysis of plasmid solution in HIC after precipitation with 2.5M ammonium sulphate. In this figure it can be verified two peaks. The first pick (20 to 29 fractions) corresponds the unretained fraction, the plasmid DNA. The fractions were collected and analysed by using HPLC. The analytical chromatogram (not results shows) obtained no longer shows the presence on the impurity peak, indicating an improvement in HPLC purity (to 100%). The second peak corresponds to the host impurities such as RNA, gDNA, denatured plasmid DNA and proteins eluted after the plasmid. Agarose gel electrophoresis also analyzed the complete removal of RNA (not results shows) and the absence of gDNA (Figure 2) for all the concentrations of ammonium sulphate used. Genomic DNA from *E. coli* is double-stranded, but becomes mostly single-stranded during alkaline lysis. During this process, the complementary strands of gDNA are completely separated and partially cleaved. The resulting DNA molecules show a high exposure of the hydrophobic bases and can thus interact with the HIC ligands (Diogo *et al.* 2000). According to the specifications, gDNA contamination should be plasmid <0.1 ng μg^{-1}.

Plasmid molecules did not interact with the HIC column, eluting in the flow through. The reason for this behaviour is that, in double-stranded plasmid molecules, the hydrophobic bases were packed and shielded inside the helix and thus hydrophobic interaction with the support ligands was minimal. Previous studies by Diogo et al. (1999) with the same HIC gel demonstrated that is also possible to separate denatured plasmid from native plasmid. Because denatured forms of contain large stretches of single-stranded DNA, there is more exposure of hydrophobic bases and,

consequently, the hydrophobic nitration is greater and retention time is longer. In fact, the HIC support studied by Diogo et al. (2000) was capable of removing denatured plasmid variants that are usually produced with the widespread method of alkaline lysis of plasmid isolation. This work also indicates the ability of the HIC support to separate proteins and genomic DNA from plasmids.

Acknowledgements

The authors acknowledge Dr. Joaquim M. S. Cabral (Instituto Superior Técnico, Lisbon, Portugal) and Dr. Ernesto Marques Júnior (The Johns Hopkins University School of Medicine, Baltimore, United states) kit supply and the plasmid for accomplishment of this work. This work was supported CAPES, FINEP, EC Alfa Program BIOENGE Network/ALR/B7-3011/94.04-6.0154.9, Universidade Federal de Pernambuco and Universidade Federal Rural de Pernambuco.

References

Diogo MM, Queiroz JA, Monteiro GA, Prazeres DMF (1999) Separation and analysis of denatured plasmid forms using hydrophobic interaction chromatography. *Anal. Biochem.* **275**:122-124.

Diogo MM, Queiroz JA, Monteiro GA, Ferreira GNM, Martins SAM, Prazeres DMF (2000) Purification of a cystic fibrosis plasmid vector for gene therapy using hydrophobic interaction chromatography. *Biotechnol. Bioeng.* **68**:576-583.

Little-van den Hurk SVD, Braun RP, Babiuk LA (2000) *Veterinary DNA vaccines*. In: DNA Vaccines: Methods and Protocols, Lowrie DB, Whalen RG (eds). Human Press, Totowa.

Prazeres DMF, Schulep T, Cooney CL (1998) Preparative purification of supercoiled plasmid DNA using anion exchange chromatography. *J. Chromatogr. A.* **806**:31-45.

Prazeres DMF, Monteiro GA, Ferreira GNM, Cooney CL, Cabral JMS (1999) Large-scale production of pharmaceutical-grade plasmid DNA for gene therapy: problems and bottlenecks. *Trends Biotechnol.* **17**:169-174.

Rhoderick EB, Jarvis KL, Hyland KJ (1989) Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal. Biochem.* **180**:136-139.

Ribeiro SC, Monteiro GA, Cabral JMS, Prazeres DMF (2002) Isolation of plasmid DNA from cell lysates by aqueous two-phase systems. *Biotechnol. Bioeng.* **78**:376-384.

Sambrook J, Fritisch EF, Maniatis T (1989) Molecular cloning: laboratory manual. New York: Cold Spring Harbor Laboratory Press.

Table 1. Purification of pVax-LacZ plasmid

Process step	Plasmid mass (mg)	Purity (%)	Purification factor	Yield (%)
Lysis	8716	5.7	1.0	100
Isopropanol precipitation	4219	6.7	1.7	48
$(NH_4)_2SO_4$ precipitation 2.0M	1837	26	3.1	69
2.5M	1490	32	2.4	53
3.0M	1388	37	2.2	49
HIC 2.0M	1202	100	3.2	40
2.5M	1229	100	3.3	51
3.0M	1184	100	3.1	53

Results reported are the average of two independents experiments and errors were judged 5% of the mean value.

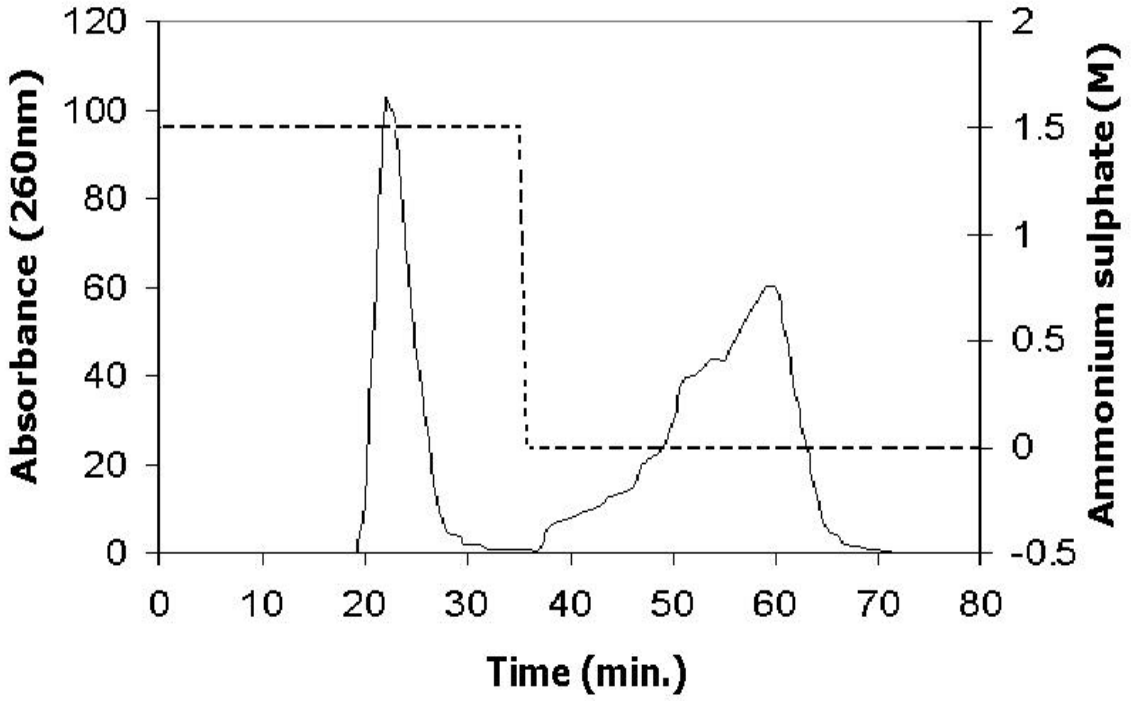
Absorbance (260nm)
120
100
80
60
40
20
0
2
1.5
1
0.5
0
-0.5
Ammonium sulphate (M)
0
10
20
30
40
50
60
70
80
Time (min.)

Figure 1

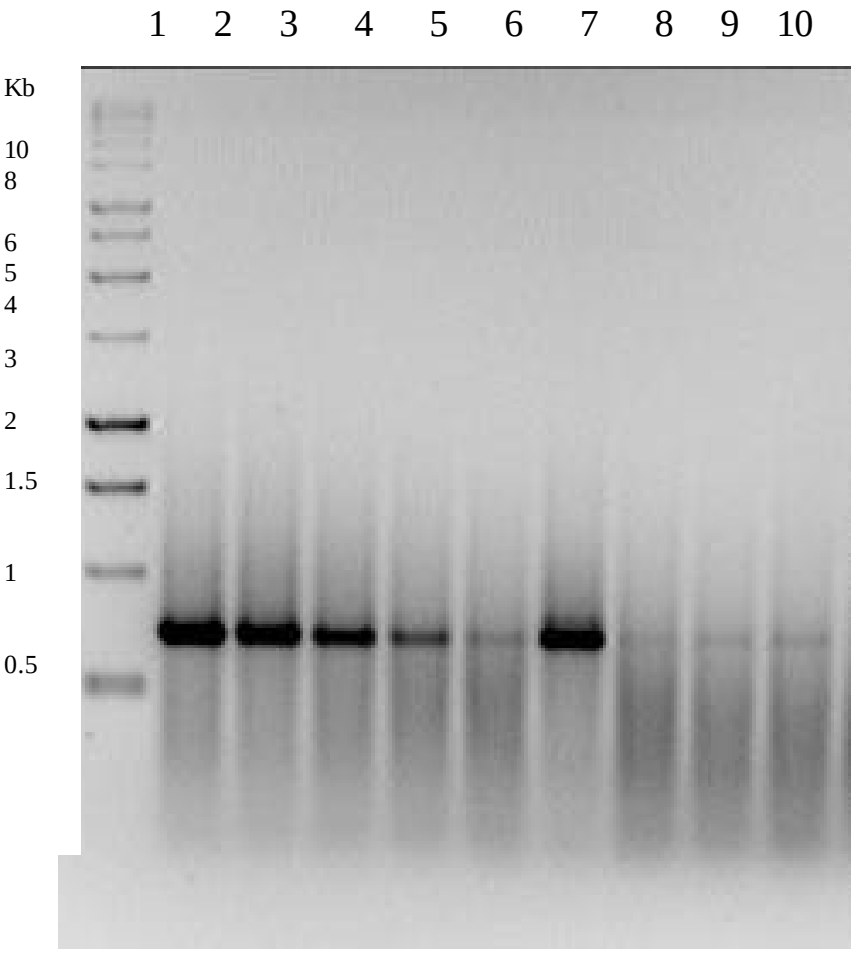

Figure 2.

Figure caption

Figure 1. Hydrophobic interaction chromatography performed on plasmid solutions after 2.5M ammonium sulphate precipitation. Elution was isocratic with 1.5M ammonium sulphate in 10 mM Tris-HCl (pH 8.0) at a low rate of 1ml/min.

Figure 2. Analysis of gDNA by agarose gel eletrophoresis: molecular weight markers (lane 1). Calibration curve (lane 2-6). Lysis solution (lane 7). Samples after HIC 2.0M, 2.5M and 3.0M precipitation with sulphate of ammonium (lane 8-10).

7. CONCLUSÕES

Os resultados experimentais obtidos permitiram as seguintes conclusões:

- A produção DNA plasmidial em agitador orbital foi dependente das condições de cultivo. A diferentes concentrações da pressão seletiva (canamicina) influenciou a produção de DNA plasmidial. Concentrações entre 30-50µg ml^{-1} demonstraram comportamento similar para todos os ensaios de produção biomassa e DNA plasmidial. Como também as diferentes velocidades de agitação usadas=. O meio de cultura TB apresentou ser o mais eficiente em termos de produção de DNA plasmidial e apresentou ser mais estável quando comparado como meio de LBG.

- O acréscimo da quantidade de solução de lise aplicado a cada sistema não é muito vantajoso para as fases analisadas (PEG 300 e 400 fase superior e PEG 1000 fase inferior), o rendimento não é proporcional à quantidade de lisado celular carregado. O sistema que apresentou melhor rendimento (37,18%) foi o sistema PEG 400 com 60% de lisado celular carregado, porém obteve um alto rendimento de proteína. O menor rendimento em proteína foi verificado com o sistema PEG 1000 com 20% de lisado obtendo 4% de proteína e rendimento em DNA plasmidial de 10,5. Sistemas com pesos moleculares menores (300 e 400) ocorreu partição do DNA plasmidial para a fase superior, enquanto sistemas com pesos moleculares maiores (1000 e 8000) ocorre partição para a fase inferior e o sistema com peso molecular intermediário (550) ocorreu partição para a interfase. O RNA preferencialmente particionou-se para a fase onde o DNA plasmidial foi particionado.

- A combinação da técnica de precipitação com sulfato de amônio e a cromatografia de interação hidrofóbica utilizada foi eficiente para purificar DNA plasmidial. Obtendo 100% de pureza para todas as concentrações de sulfato de amônio após sua eluição na matriz cromatográfica e fator de purificação de 3,3 na concentração de 2,5M de sulfato de amônio.

8. SUGESTÕES PARA ESTUDOS FUTUROS

Como todo trabalho de pesquisa, este segue a regra, uma vez que é praticamente difícil a obtenção de um trabalho totalmente completo, e torna-se importante a apresentação de algumas sugestões para que possam contribuir para o melhor as condições de produção e purificação de DNA plasmidial, propõe-se:

- Realizar estudos de produção de DNA plasmidial com diferentes variáveis em fermentador com auxílio de planejamento estatístico fracionado ou completo;
- Analisar a eficiência de resinas de caráter hidrofóbico visando aumentar o rendimento em DNA plasmidial;
- Verificar o comportamento de partição do DNA plasmidial no sistema PEG/citrato;
- Investigar a formação de isoformas do DNA plasmidial durante os processos de lise celular e na partição em sistemas de duas fases aquosas.

9. ANEXOS

Os resultados obtidos neste trabalho foram apresentados em congressos nacionais e internacionais.

1. Resumo aceito para apresentação no XXII Congresso de Microbiologia: Influência das condições de cultivo na estabilidade do plasmídeo pD2 em *Escherichia coli.*

2. Resumo apresentado no 12th International Conference on Biopartitioning and Purification: A preliminary study of dengue 2 plasmid DNA vaccine (pD2) extraction from cell lysates by aqueous two-phase systems.

3. Resumo apresentado na XXXI Reunião Anual da Sociedade de Bioquímica e Biologia Molecular. Effect of antibiotic concentration and culture aeration on pVaxDN2 production *E. coli*.

4. Resumo apresentado na XXXI Reunião Anual da Sociedade de Bioquímica e Biologia Molecular. Plasmid stability pVaxDN2-carring *E. coli* during batch-fermentation.

5. Resumo apresentado na XXX Reunião Anual da Sociedade de Bioquímica e Biologia Molecular. Partitioning and purification of plasmid DNA in aqueous two-phase systems.

Durante a realização deste trabalho foram realizadas atividades paralelas, os resultados obtidos foram submetidos para publicação em periódicos internacionais e apresentados em congressos nacionais e internacionais.

1. Characterization of protease from *Penicillium aurantiogriseum* for application in food industries. 4th European Congress Engineering, 21 a 25 de setembro, Granada, Espanha, 2003 .

2. Tratamento enzimático utilizando proteases de *Nocardiopsis* sp. em águas residuais de uma indústria de laticínios. XXII Congresso Brasileiro de Microbiologia, 17 a 20 de novembro, Florianópolis, SC, 2003.

3. Otimização da produção de proteases produzidas por *Candida butyri*. XXII Congresso Brasileiro de Microbiologia, 17 a 20 de novembro, Florianópolis, SC, 2003.

4. Study of the conditions of production of proteases for *Aspergillus sydowii*. VII Simpósio de hidrolise enzimática de biomassas, 06 a 10 de dezembro, Maringá, PR, 2002.

5. Use of aqueous two-phase systems on perforated rotating disc contractor for continuous extraction of *Schitosoma mansoni* recombinant antigen. XXXI Reunião Anual da Sociedade de Bioquímica e Biologia Molecular, 18 a 21 de maio, Caxambu, MG, 2002.

6. Partial purification on inulinase from *Aspergillus niveus*. XXX Reunião Anual da Sociedade de Bioquímica e Biologia Molecular, 19 a 22 de maio, Caxambu, MG, 2001.

7. Partitioning and purification of monoclonal antibody of *Toxoplasma gondii* in PEG/potassium phosphate aqueous two-phase systems. XXX Reunião Anual da Sociedade de Bioquímica e Biologia Molecular, 19 a 22 de maio, Caxambu, MG, 2001.

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PRODUCTION OF HYDROLYTIC ENZYMES TO FORMULATE BIODEGRADABLE DETERGENTS

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The development and commercial use of biodegradable detergents have rapidly increased the demand for mass and inexpensive production of hydrolytic enzymes, chiefly proteases, lipases, amylases and cellulases. This category of detergents provides energy saver and lower cost equipment since the process abolishes the need for resistance to heat, pressure and corrosion. In addition, this new generation of detergents, without phosphate and chlorine whiteners, provide a major environmental advantage since all components are biodegradable and allow safer and less caustic formulations. The Brazilian market imports increasing quantities of such enzymes and even ready to use formulations. This work is a collaborative effort between a RS State based industry (TECFARM) and CBIOT (UFRGS) to establish cost effective large-scale production of the desired hydrolytic enzymes and to develop and test formulations aiming maximum activity and storage time. We have initially selected some bacterial and fungi strains producers of amylases and proteases. Using an eleven litres reactor we adapted the cultivation conditions to optimise the production and secretion of the desired enzymes using low cost substrates. The enzymes up to now produced (amylases and proteases) were used to formulate detergents and their effectiveness and enzyme activities were monitored under laboratory and routine use conditions. Commercially available detergents were used as standards. We found that the enzymes, up to now produced, match the activity of commercial enzymes and are cost effective.

Supported by: FAPERGS, TECFARM, CNPq.

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FLAVOR ESTER SYNTHESIS CATALISED BY *Mucor miehei* LIPASE IN SOLVENT - FREE MEDIUM

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Esters of short-chain fatty-acids and alcohol are important natural aroma compounds used in the food industry to enhance the flavours. A great number of these esters are responsible for many fruit aromas, as banana, pineapple and strawberry. Such esters, when produced by chemical ways, are not considered natural aromas. On the other hand, food aromas obtained by biotechnological ways are classified as natural by the European and US food legislation. Microbial lipases have showed great potential for industrial applications as catalysts in synthesis reactions. However the systems are usually made in organic solvent media. In this work we tested different ester synthesis using *Mucor miehei* lipase in a non-organic solvent system. The reaction mixture consisted of 0.02M fatty acid, 0.04M alcohol, 0.8g molecular sieves and 1g% *Mucor miehei* lipase.
The results showed that *Mucor miehei* lipase was able to catalyse ester synthesis reactions with a high yield (98%). The efficiency in the synthesis reaction was higher when short chain organic acids were used as compared with long chain ones. In our conditions, the best results were obtained for isoamyl butyrate synthesis. Studies about the components concentration in the reaction showed that 2,5 mM butyric acid and 45 mM isoamylic alcohol are the better conditions for isoamyl butyrate synthesis.

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PEPTIDE BOND FORMATION CATALYZED BY PANCREATIC PORCINE LIPASE IN BIPHASIC ORGANIC MEDIUM

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As lipases are not expected to exhibit amidase activity, we decided to investigate the feasibility of using them to catalyze kinetically controlled peptide segments condensations. Thus, we first performed a systematic study of the coupling between Ac-Tyr-OEt and Gly-NH_2 mediated by a commercial pancreatic porcine lipase from Sigma (cPPL)[1]. The yield obtained 90% in 5-minute incubation carried-out under optimized reaction conditions, but part of the Ac-Tyr-Gly-NH_2 formed was hydrolyzed in longer-lasting reactions (40% of the total at 72h). To disclose the presence of proteases in the cPPL preparation we then analyzed it by PAGE/SDS. As expected, α-chymotrypsin was identified as the major contaminant. Irreversible inhibition with TPCK was monitored by the measurement of the capability of the treated cPPL to hydrolyze the ester of Ac-Tyr-OEt. As this ability was greatly reduced after 2h, the enzyme solution was dialyzed against water and lyophilized. The resulting powder catalyzed a comparative peptide bond formation trial conducted under the optimized reaction conditions previously employed. The coupling yield found in a 5-minute incubation was 50%, going up to 70% in 72h. No secondary hydrolysis was observed. Spontaneous hydrolysis of Ac-Tyr-OEt was also monitored, showing to be less than 1% in 24h. The most appropriate cPPL concentration for efficient dipeptide formation was determined as well. This varied from 0.1 to 0.2 mM. These results revealed that the commercial cPPL picked is a very potent and cheap catalyst of enzymatic dipeptide synthesis. However, for peptide fragment condensations that must be previously purified or treated with TPCK. As far as we know, we are the first to unequivocally show that: 1) the contamination by proteases in the commercial cPPL indeed interfere with its esterase activity; 2) this fact may have important implications in amino acid and peptide chemistries.

Lirla, C.W. & Miranda, M.T.M., Peptides 2000 (in press).

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PARTITIONING AND PURIFICATION OF MONOCLONAL ANTIBODY OF *TOXOPLASMA GONDII* IN PEG/POTASSIUM PHOSPHATE AQUEOUS TWO-PHASE SYSTEMS

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The Toxoplasmose is a congenital and acquired disease caused by a protozoal infection. The symptoms of acquired form are fever and lymphadenomegaly and the symptoms of congenital form are intracerebral calcifications and chorioretinitis of the newborn. Due to these symptoms, it is necessary a previous diagnosis, to start the correct treatment as soon as possible and avoid unnecessary treatment. Nowadays, the usual methodology is ELISA (enzyme linked imunoadsorbation assay) which needs specialized people and has a high cost. Two-phase aqueous systems provides a mild method for purification of proteins, and scale up to large volumes presents no engineering problems. This work has the proposal of utilization of two-phase aqueous systems polyethylene glicol (PEG)/phosphate salt to extraction and purification of monoclonal antibody against **Toxoplasma gondii** produced by the tachyzoite form. Aqueous two-phase systems of total mass of 3g were prepared by weighing appropriated amounts of concentrated solutions of PEGs (1000 and 8000) and by adding K_2HPO_4 and KH_2PO_4 solutions until required pH value was obtained. The detection of antibody in both phases were determined by dot-blotting. The antibody partitioned by PEG 1000 was found mainly in the PEG-rich phase for the smaller tie line at pH 7.0. However the best purification was reached in PEG 1000/potassium phosphate system for the higher tie line.

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A HARDWARE/SOFTWARE ARCHITECTURE FOR DATA ACQUISITION IN BIOSENSORS FOR IMMUNODIAGNOSTICS

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This work describes the development of a hardware/software architecture for data acquisition in biosensors for immunodiagnostics. This approach has been used to determine humoral response against *S. pneumoniae* after vaccination. It was utilized a quartz crystal microbalance, 10MHz, in a FIA system. A laboratory model without connection with the patient was used (in vitro). This approach was based on the Hardware/Software platform, denominated CHAMELEON-I, composed basically of a FPGA (Field Programmable Gate Array), a microcontroller, RAM and EEPROM memories, oscillators and conditioners of signs. The FPGA is responsible for two stages of the signal treatment. In the first stage, the FPGA acquires the signals from the biosensor in a second stage of the process of handling of the data, the FPGA will be configured to serve as a co-processor for calculation of system relaxation. The communication with the PC and reconfiguration of the FPGA during the two stages of aquisition/treatment data between memory and FPGA for relaxation calculation are done by the microcontroller. A supervisory system, installed in PC, provides commands that allow the system controls all operation cycle of the system, makes the sampling of the data to the user in form of tables and graphics in time of execution. This methodology, based on FPGAs, allows and easy and fast adaptation of calculation and acquisition methods, with few changes of the hardware platform. Thus, low cost platforms can be easily developed for different biosensors data acquisition approaches.

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AN UNIVERSAL AMPEROMETRIC IMMUNOSENSOR OF GLASS BEADS USING A MODIFIED GRAPHITE-EPOXY ELECTRODE IN FIA SYSTEM

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The biosensors are faster, cheaper and safer than the conventional methods of clinical analysis and also to many cases sensible and portable. The optical and piezoelectric technologies are more sensitive and faster to the immunosensors development, but the non-specific binding represents its major limitation. In despite, the amperometrics are more selective and involve electronic easier sensors. This work purposes a changeable reactor filled with immobilized glass beads using the same electrode as sensor for different diseases. Anti-human-IgG conjugated to peroxidase (Anti-hIgG-POX) was immobilized on glass beads as model and the amperometric signal was generated by peroxidase reaction into a modified epoxy-graphite electrode in the FIA system. The glass beads, after cleaned with methanol, were pre-activated using polietyleneimine (2%) to perform a thin-film for covalent immobilization. The working electrode was prepared by mixing epoxy, graphite, silver, tetracyanoquinodimethane and the peroxidase enzyme compounds followed by curing for 72 h at 30°C. This modified biocomposite electrode was disposed in the chamber reaction against a reference electrode at 0.26V. The FIA system was carried using 0.1M tris-citrate buffer (pH 6.0); flow rate around 600µl/min. Different dilutions of Anti-hIgG-POX immobilized on glass beads reactor were tested from 0.1µg/ml to 2µg/ml, and after injection of 50mM hydrogen peroxide. The amperometric response was linear from 0.2µg/ml to 2µg/ml showing a correlation coefficient of 0.997 ($p<0.05$). These results point a new method immunosensor that can be used to detect different immunocomplex by changing only the active receptor immobilized on glass beads reactor.

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IMMUNOCHEMICAL CHARACTERIZATION AND BIOLOGICAL ACTIVITY OF *A BORDETELLA PERTUSSIS* ADJUVANT PREPARATION

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Interest in the development of adjuvants is growing rapidly since many new vaccine candidates have emerged over the last years. The limitations of aluminum compounds lead us to the search of alternative adjuvants for use in humans and one which *B. pertussis* is a promising candidate. We have been working on the immunomodulatory properties of a *B. pertussis* soluble fraction (BS) obtained from bacterial cell mass. We have shown that BS can increase the antibody response to a variety of unrelated antigens. In order to identify and characterize the components involved in the modulation of the immune response BS was submitted to anion exchange chromatography (Mono Q-Sepharose). The fractions obtained were injected mixed with DPT (Diphteria-Pertussis-Tetanus) vaccine in BALB/c mice, and the animals were further challenged with live *B. pertussis*. The results showed that 78% of the mice immunized with the fraction eluted at approximately 350mM NaCl (25 mM Tris buffer, pH 8,0) mixed with DPT were protected against the challenge compared with 30% of mice immunized with DPT alone. We are now evaluating the antibody immune response and cytokine profile of these animals. These results confirm and extend our previous work on adjuvant properties of this soluble *B. pertussis* fraction which is a good candidate to be explored for inclusion in vaccination protocols.

FAPESP, FUNDAÇÃO BUTANTAN

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SOMATIC EMBRYOGENESIS AND GENETIC TRANSFORMATION OF CASSAVA CULTIVARS FROM THE NORTH-EAST OF BRAZIL

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The very low amount of protein in the roots of cassava (*Manihot esculanta* Crantz, Euphorbiaceae), is the main constraint in its wide use as animal food. Genetic engineering techniques have the potential to overcome this constraint. Recent developments in cassava biotechnology, such as the availability of regeneration and transformation protocols for model cultivars, prompted us to develop methods for cassava regeneration and transformation for the six most widely used cultivars in the Brazilian north-east (Água Morna, Ananás, Burro, Branca, Preta, Tapioca, Rosa and Rosinha). The shoot apex from *in vitro* grown plants, were isolated and incubated in the dark in Petri dishes containing MS medium supplemented with picloram in the range from 1 to 10 mg/L. After three weeks of incubation, somatic embryos were induced at each picloram level tested, but the frequency of embryogenesis was higher at the concentration of 9 mg/L. The primary embryos thus obtained were matured by transfer to MS medium supplemented with BA at 0,4 mg/L, under light conditions and after embryo maturation, pieces of green cotyledons were transferred to Petri dishes containing picloram at 9 mg/L, in order to induce secondary embryogenesis. The green cotyledons from the somatic embryos were cut into pieces and co-cultivated with *A.tumefaciens* LBA4404 harbouring the plasmid pPARGUSII, in which the root-specific promoter PAR controls the expression of the gene *uidA*. After four days of co-cultivation, the integration of the *uidA* gene into the genome of the cultivars tested was demonstrated by histochemical analysis. We are now using these protocols to obtain transgenic lines of the varieties Rosinha and Água Morna that are being transformed with a gene for an arbitrial reserve protein (ARP), put under the control of the root specific promoter PAR.

Financial support: Centro Brasileiro-Argentino de Biotecnologia, CNPq and FUNCAP

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PLASMID STABILITY pVax-IN2-CARRYING *E. coli* DURING BATCH FERMENTATION

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Plasmid DNA has recently acquired considerable interest due to its attractive potential application in gene therapy and DNA vaccines (Prazeres *et al.*, 1999). DNA vaccines can be developed from a pathogen's genes to provide immunity against diseases. Plasmids for gene therapy containing human or nonhuman genes are very large molecules in comparison to proteins. Our knowledge about culture media for the cultivation of recombinant *Escherichia coli* has been obtained, which optimised the expression of proteins. However, environmental and nutritional conditions for optimisation of plasmid DNA production *E. coli* could be significantly different from those of protein production. In this work we are investigating plasmid stability of a recombinant *E. coli* expressing protein which can be used as a vaccine. We are focusing on the plasmid stability pVax1IN2-carring *E. coli* was grown in TB medium with kanamycin (50 mg/ml) selective marker, grown in a 37°C orbital shaker at 120, 160 and 200 rpm during 8 hours. The growth was followed by optical density at 600nm, dry cell weight, cell counting and purified plasmid DNA. Plasmid DNA was purified with FlexiPrep mini kit (Pharmacia). The plasmid instability was measured by the rate of plasmid loss when the recombinant strain was grown in the absence of kanamycin. The specific growth rates 0,41 h^{-1} for the culture at 160 rpm, obtained 190 mg/ml in culture growth for 6 hours. The plasmid instability of pVax-IN2 on TB medium was 19%.

Financial Support: CAPES, UFPE, CNPq

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EFFECT OF THE SULPHATED α-GLUCAN FROM *RAMALINA CELASTRI* AGAINST EXPERIMENTAL SCHISTOSOMIASIS: PRODUCTION OF SPECIFIC IgG AND EFFECT AGAINST SPLEEN AND INTESTINAL HISTOPATHOLOGICAL INJURIES

Araújo, R.V.S.[1], Pinheiro, A.F.A.[1], Souza-Araújo, F.V.[1,2], Lopes, S.L.[3], Santana, J.V.[1,4], Mattapallil, E.[1,5], Gomes, P.A.J.[6], Iacomini, M.[6] and Carneiro-Leão, A.M.A.[1,2]
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The sulphated derivative of the (1→3), (1→4)-α-D-glucan from the lichen *Ramalina celastri* (α-Glu-SO$_3$) has showed to be effective against the liver injuries caused by *Schistosoma mansoni* severe and mild infections (Procópio *et al.*, XXIX SBBq, L-20, 2000; Araújo *et al.*, XXX SBBq, H-23, 2001). The aim of this work was to observe the effect of α-Glu-SO$_3$ against spleen and intestinal injuries and on the α-SWAP antibodies levels (IgG) in infected mice. Four groups (n=15/group) composed by *S. mansoni*-infected female Swiss mice (BH strain, caudal infection) were treated with a single i.p. dose of the drug 24 hours after infection, as follows: G-1, infected with 70 cercariae/animal and treated with α-Glu-SO$_3$ (10 mg.Kg^{-1}); G-2, 70 cercariae/mouse, treated with NaCl 150 mM; G-3 and G-4, infected using 150 cercariae/animal and treated with α-Glu-SO$_3$ and NaCl 150 mM, respectively. The animals were killed at 6th, 7th and 8th weeks after the infection, when blood, spleen and intestines were collected. Tissues were were fixed in formaldehyde 4% v/v 0.01 M PBS and processed for HE staining. Blood was tested by ELISA against SWAP (Soluble Worm Antigen Protein). Histopathological analysis did not show any statistically significant difference between treated and control groups. It was also observed an increase of IgG levels along the infection. These results are possibly related to the immunomodulator activity of polysaccharide.

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Site-directed mutagenesis of bovine FGF-2 cDNA allows the production of the human-form of FGF-2 in *Escherichia coli*

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Therapeutic angiogenesis is a new promising strategy that has been proposed in the past few years for the treatment of different diseases resulted from ischemias as well as deficiencies in wound healing. Although the knowledge in this area is still in the beginning, the results accumulated from a variety of works point to Fibroblast Growth Factor as a good candidate for this therapy. Here we describe a site-directed mutagenesis of bovine FGF-2 cDNA in order to produce the human-form of FGF-2 (FGF-2H) in *E. coli*. The recombinant protein was expressed in *E. coli* and displays in vitro activities measured by proliferation of A31 fibroblast cells and neuronal differentiation of PC12 cells. FGF-2H was also able to induce wound healing in rabbit ear dermal ulcers and histological analyses showed induction of angiogenesis and fibroblast proliferation, but incomplete re-epithelization. The expression of recombinant FGF-2H can now be scale up, aiming the production of the human-form of FGF-2 under Good Manufacturing Practices (GMP) suitable for clinical trials.

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EXPRESSION OF CTB:PSAA FUSION PROTEIN IN *E. coli* FOR VACCINATION AGAINST *Streptococcus pneumoniae* INFECTION

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Cholera toxin B subunit (CTB) is the non-toxic portion of cholera toxin (CT). CTB is responsible for the holotoxin binding to the GM1 ganglioside at cellular surface which is further endocytosed. CTB has been described as a mucosal adjuvant for oral and nasal vaccines.
PsaA is a conserved surface adhesin protein of *Streptococcus pneumoniae*. It has been shown that this protein elicits protection against colonization of the pathogen. The aim of this work is the expression of a fusion protein CTB-PsaA in *E. coli*, in order to improve the mucosal response in the upper respiratory tract against *Streptococcus pneumoniae*.
The psaA gene was amplified by PCR and the correct sequence was confirmed by DNA sequencing. The pAE ctxB construct previously obtained was digested and the psaA was cloned in the 3' terminus of the ctxB gene. The pAE is an *E. coli* vector that uses T7 promoter. In this system the protein can be expressed with a 6Xhis tag in the N-terminus, in order to facilitate the purification in a Ni^{2+} - charged chromatography column. *E. coli* BL21 (SI) competent cells were transformed with the pAE-ctxB-psaA construct. In this strain, the T7 RNA polymerase is under the control of the osmotically inducible *proU*, therefore the induction was performed by the addition of NaCl. The recombinant protein was extracted from the bacterial soluble fraction and purified. The functional assay in Y1 cells and immunological characterization are underway.

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PROTEASE PRODUCTION BY YEASTS ISOLATED FROM RAW COW MILK

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Yeasts are unicellular moulds used in the proteases production of industrial interest. Proteases are enzymes with great variety of applications, mainly in industries of detergents and foods. The aim of this work is to isolate yeasts from raw milk samples of cow and to selection species producing of proteases. The isolation was carried out in MRS (De Man, Rogosa and Sharpe) and APT agar culture media, using as samples raw milk of cow in three different volumes (25, 50 and 100 ml) with five dilutions each (1:10 up to 1:100.000). The cultures were isolated in the following conditions: temperatures 30° C and 37° C, incubation time of 48 hours. Ten yeasts were identified for accomplishment for proteases production applying qualitative and quantitative tests. For the qualitative test, the yeast growth in gelatin-Mannachini broth and assayed using agar-gelatin-milk, (specific medium for production of proteases). The quantitative test was carried out according to Leyton, 1979, that it uses the azocasein with substrate. The protein total determination was according to Bradford, 1976. The best results from qualitative test, were obtained with yeasts Y18, Y10, Y22, Y24 and Y27 (18, 19, 22, 18 and 20 mm zone diameter, respectively). Higher yield protease activity was observed using the isolated Y27, with total proteolytic activity of 610U/L and specific proteolytic activity of 23.5 U/mg. These results suggest that the isolated Y27 its potential source of proteases for industry.

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Kluyveromyces marxianus CBS6556 GROWTH IN RESIDUES FROM A SUCROSE-RICH SYRUP FACTORY

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Brazil is one of the most important producers of sugar cane in the world, but the sugar manufacturing results in some agro-industrial residues, like bagasse and molasse. The sucrose-rich syrup was produced in a sugar factory by additional steps of filtration with the purpose of obtain a clear and much pure product than the tradiotional procedures. But, this process generates residues with high sugar concentration, responsable for high biological oxygen demand. The utilization of these residues for single cell protein production has been analyzed. The growth of *Kluyveromyces marxianus* CBS6556 was performed at 28°C, 180rpm for 24 hours and the samples harvereste were analysed for sugar consumption by DNSA method and cells by Neubauer chamber counting procedure. Using the supplemented residues as a medium, comparing to *Saccharomyces cerevisiae* Itaiquara growth, the *K. marxianus* had a better development. Then, the performance of this strain was observed in sucrose media, analysing the importance of each supplementation for the yeast growth. The results demonstrate that supplementation with phosphorous source in defined medium can improve the development of this strain, the opposite of the results were observed in the tests with the sucrose-rich syrup residues. The growth observed in both media was quite similar, this can indicate that the residue has no toxic components. The sucrose consumption rised to 70% after 24h, that was lower than observed in the residue growth. The SCP production, with *Kluyveromyces marxianus* growing in sucrose-rich syrup residues, can be improved in future studies by combination of different parameters of fermentation.

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INFLUENCE OF AERATION ON LIPASE PRODUCTION BY *Yarrowia lipolytica*

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Lipases have been produced by different microorganisms, under different conditions. The interest on these enzymes increased considerably due to their versatility, specially for organic synthesis. We have previously investigated lipase production on shaken flasks and bench-fermenter under different agitation speeds. In the present work, the influence of aeration on lipase production by *Yarrowia lipolytica* was studied in bench fermenter, at 200 rpm with air flows of 1, 2 and 3 dm^3/min. Protease, lipids, nitrogen and pH were also measured during cell growth.

An anticipation of maximum lipase activity levels was observed when aeration was increased. In opposition, increase of aeration led to a decrease of maximum lipase activity levels, production, $Y_{P/S}$, $Y_{P/X}$. Higher air flows (2 and 3 dm^3/min) resulted in distinct lipid consumption rates, while total nitrogen concentration decreased, with distinct consumption profiles and protein release to the culture media, in each case. Similar pH profiles were seen under these different conditions, presenting an initial decrease, followed by an increase at late stationary phase, after a period of stabilization. It was also noticed that k_La was more affected by agitation speed than by aeration. These results reinforce the strong dependence among oxygen transfer, lipase production and cell metabolism, as suggested previously.

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EFFECT OF ANTIBIOTIC CONCENTRATION AND CULTURE AERATION ON pVaxDN PRODUCTION *E. coli*

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A study of antibiotics minimum concentration in the plasmid DNA production is one of the main spot at the final cost of the wanted product. The pVaxDN2 is one plasmid which has a good potential for DNA vaccine. The optimum conditions of cell growth and plasmid production were carried out together with different concentration of kanamycin related with the aeration conditions. The fermentations, were supplemented with different concentrations of kanamycin (10, 20, 30µg/ml), used the medium for innoculum and growth, the cells were incubated for 8 hours at 37°C in an orbital shaker at 120, 160 and 200 rpm for 8 hours. Samples of 1 ml have been taken out, and biomass and plasmid quantification were carried out measuring the absorbance at 600 and 260nm respectively. The results suggested that the optimal conditions for growth of the recombinant producing strain are: concentration of kanamycin 10 mg/ml, with a maximum growth of 0.5 h^{-1} and best production speed of the 200rpm.

Financial Support: CAPES, FINEP, CNPq

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Kluyveromyces marxianus CBS6556 GROWTH USING "COALHO" AND RICOTTA CHEESE WHEY AS SUBSTRATE FOR SCP PRODUCTION

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The cheese whey is a by-product of dairy industry that represents a potential pollutant due to the high lactose concentration. For degradating this sugar is necessary the galactosidase enzyme produced by some microorganisms like *Kluyveromyces marxianus* yeast, considered GRAS by FDA and already used to produce single cell protein (SCP). The growth and the enzyme production in *K. marxianus* CBS6556 was observed in "coalho" and ricotta cheese whey. The strain was cultivated at 28°C, 180 rpm for 24 hours and the samples were looken and analysed for β-galactosidase enzyme activity, growth by counting cells in Neubauer chambre and sugar consumption by DNSA method. The substrates were "coalho" whey and ricotta whey from a cheese factory in Cachoeirinha – PE, autoclavated for 15 minute at 121°C. The CBS6556 showed a long lag phase and slow growth in the first 16 hours, and the log phase can be observed after 20 hours of cultivation, and no stationary phase was detected only in these substrates. The sugar consumption was more than 80 percent, and the enzyme production rised to 4.800 U/mg of protein, after 32 hours. These results demonstrate that this yeast can be used as SCP production, and its growth can be optimized by fermentation parameters.

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EFFECTS OF THE CELL HYDROPHOBICITY AND CHARGE ON YEAST CELL FLOTATION

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Flotation is a separation process based on the affinity of hydrophobic cells to air bubbles. The yeast cells (*Saccharomyces and Hansenulla*) were grown in agitated cultures of molasses and defined medium for the determination of the flotation in a bench flotation column (deSousa and Laluce, *Biotechnol. Lett.* 22: 753, 2000). The cell hydrophobicity was determined by a method (Straver and Kijne, *Yeast* 12: 207, 1996) based on the affinity of the cells to supermagnetic latex beads from Sigma (act 27H0890) or by the method based on the partition of the cells between the medium and the hexane phases (Romano et al, *Colloids Surf. B* 2: 511-515,1994). The measurement (based on the migration of cells in an electrical field) of the zeta potential was carried following the procedure describe by Deo and Natarajan (*Minerals Eng.*, 12: 1339, 19 in a Zeta Pals apparatus from Brookhaven (USA). Only the yeast cells showing levels of hydrophobicity were capable of efficient flotation. The adhesion to magnetized beads showed to be a better procedure for the measurement of hydrophobicity. The rises in pH of the media during growth had more affect on flotation efficiency (enrichment of the cells in the foam than the flotation (total recovery from the medium). The zeta potential changed with the media. In addition strain FLT-01 of *Saccharomyces cerevisiae* showed a high negative value of potential at pH above the cellular PI while very low values of hydrophobicity obtained with *Hansenulla*. However, both yeasts were highly hydrophobic and show high flotation capacity. Lastly, flotation and the hydrophobicity capacities seem not dependent on the charge and hydrophobic interactions at the interfaces (medium/cells/air bubbles) than on the absolute values of hydrophobicity and cell charge.

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A study preliminary of dengue 2 plasmid DNA vaccine (pD2) extraction from cell lysates by aqueous two-phase systems

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The widespread application of recombinant DNA techniques and the rising interest in gene therapy has increased the need to develop large scale DNA purification methods consistent with the regulatory agencies guidelines. Aqueous two-phase systems (ATPS) have a widespread use in biochemistry and biotechnology for purification of biological materials. These systems are suitable for purification of biological material as the phases contain 80-95% water, thus reducing the risk of denaturation of labile biomolecules. However, the use of this method to purify plasmid DNA has evolved very little and few related references have appeared in the literature in the last years. In this work we are investigating of the partitioning in PEG/K_2HPO_4 systems of the dengue 2 plasmid DNA vaccine (pD2) expressing the pre-membrane and envelope proteins (preM-E) from a clarified *E. coli* alkaline lysate. The ATPS composition is the typical for the PEG molecular weights used (20%/20% w/w for systems with PEG 300, 400 and 550; 15%/13% for system with PEG 1000 and 10%/10% for systems with PEG 8000) and lysis solution 20, 40 or 60% of total mass. The plasmid DNA in the ATPS was quantified by fluorescence and was based on bicinchoninic acid (BCA) method. The plasmid partitioned by PEG 300 and 400 were found mainly in the PEG-rich, for system PEG 1000 and 8000 the partition was present in the bottom phase. The best results are in accordance with agarose gel analysis reached in PEG 300/K_2HPO_4 (20%/20%) system.

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Título

INFLUÊNCIA DAS CONDIÇÕES DE CULTIVO NA ESTABILIDADE DO PLASMÍDIO pD2 EM *Escherichia coli*

Autores

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Resumo

A estabilidade plasmidial é de vital importância em aplicações práticas das tecnologias de DNA recombinante. Os plasmídios são componentes essenciais em processos fermentativos envolvendo organismos geneticamente modificados. O presente trabalho descreve a influência de dois meios de cultura e diferentes velocidades de agitação na estabilidade DNA plasmidial. O plasmídio utilizado neste ensaio foi o pD2, plasmídio que expressa a pré-membrana e proteínas do envelope do vírus da dengue. A *Escherichia coli* recombinante foi crescida durante 18 horas no meio de cultura Luria-Bertani (LB) suplementada com 30μg/ml de canamicina em agitador orbital a 160 rpm e 37°C. Os meios de cultura investigados foram Terrfic both (TB) e Luria-Bertani com glicose (LBG), suplementado com 30 μg/ml de canamicina. O inóculo foi de 10% do volume final do meio de cultura crescido por 18 horas, em agitador orbital a 120, 160 e 200 rpm a 37°C. A estabilidade plasmidial foi avaliada segundo a metodologia de Lee et al. (1994). O plasmídio foi estável, em torno de 80%, nos dois meios de cultura utilizados, sendo 200 rpm a melhor velocidade de agitação.

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IDENTIFICATION OF HEAT SHOCK PROTEINS ON SUCEST DATA MINING

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ESTs sequencing of different plant structures are raising many information about sugarcane protein expression which need to be conveniently annotated and known very soon to give support for design experiments to better check gene expression in these organisms trying to understand since their metabolism to host-parasites relationship. Our group is very interested on identify and annotate proteins from the SUCEST database related to environmental stress as well as the heat shock proteins which can help the plant to support strong variation in temperature and metabolic changes to survive stress conditions. Heat shock genes are induced by intracellular conditions that denature proteins (such as elevated temperature, "heat shock"). Some encoded proteins that are relatively resistant to denaturing conditions and act to protect other proteins from denaturation; others are chaperonins, that refold denatured proteins. Members of the heat-shock protein 70 (Hsp70) family acts as a central players when vertebrates and flies acquire tolerance to otherwise-lethal temperatures. Bacteria and yeast, by contrast, use the Hsp100 family in this role. Since the heat shock response is one of the most highly conserved biological responses to environmental changes, our main objective is searching these genes in SUCEST, based on sequences already existing in databases from another plants as well as Arabidopsis thaliana, Zea mays, tomato, rice, Curcubita sp and others. So far, we found the follwing HSPs: LMW heat shock proteins, HSP101, HSP 70 kD, HSP 18, HSP26, 17.8 kD small HSP, HSP 90, heat shock factor 6, HSP cognate 70, HSP16.9. These results suggest that there are several kinds of different HSPs which play an important role in protecting the plant cell against damage caused by oxidative stress as well as by heat stress.

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PRODUCTION OF XYLANASE BY *PENICILLIUM JANTHINELLUM* ON OAT HUSK MEDIUM

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Hemicelluloses are low-molecular-weight polysaccharides, associated with cellulose and lignin in plant cell walls. Xylans are the major hemicellulosic components of hardwoods and graminaceous plants where they constitute about 25% of the total biomass. Softwood contains about 10% of xylans and lignocellulosic wastes contain 30-40% of hemicellulose. Their complete hydrolysis requires the action of several enzymes among that endo-β-1,4-xylanases are crucial for xylan depolymerization. Xylanases have potential applications in bread making, beer and juice clarification, partial xylan hydrolysis in animal feed and as bleaching agents in the pulp and paper industry.

After cooking, the yellow/brownish kraft pulp must be bleached before paper production. This is accomplished by removing the residual lignin in a multistage bleaching process using chlorine, chlorine dioxide and NaOH treatments. Xylanase pre-treatment seems to facilitate the subsequent chemical bleaching steps where it allows a decrease of the chlorine demand and improves the final paper brightness.

The prerequisite for the application of xylanase in the paper industry is that the enzyme preparation must be free of cellulase activity. The xylanase produced by *Penicillium janthinellum* strain contains high xylanolytic activity, but no detectable cellulolytic activity. Cultivation conditions for producing xylanase using oat husk hemicellulosic hydrolysate were investigated. High xylanase yield was obtained after 84 hours of fermentation with an initial pH of 5.1. The enzyme activity was measured using birchwood xylan as substrate. The maximal xylanase activity was 54 units/mL in 144 hours. Cell growth, total protein, acid and alkaline proteases, and pH were analysed.

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ISOLATION AND THE PARTIAL PURIFICATION AND CHARACTERIZATION OF L-ASPARAGINASE II FROM *S. cerevisiae* – A POTENT GROWTH INHIBITOR OF LEUKEMIC CELLS

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L-asparaginase is widely used as an antioncolitic agent for the treatment of acute lymphoblastic leukemia and lymphosarcoma, because it deprives the malignant cells of L-asparagine causing their death. These cells can only synthesize L-Asn slowly being dependents of an exogenous supply. In this study we worked with *Saccharomyces cerevisiae* as the source of L-asparaginase. Is well known that S. cerevisiae synthesizes two forms of the enzime: L-asparaginase I intracellular and L-asparaginase II extracellular (periplasmic) but only the second one has antilymphoma properties. We tested some methods for the enzime extraction being the Triton X-100 permeabilization method at 2,5 %(w/w) in glycine buffer pH 8,0, that which gave the best asparaginase II recovery. For the enzyme purification, two approachs were employed: one of them used anion exchange chromatography (FPLC-MonoP) and the other used hydrophobic interaction chromatography (FPLC-Phenyl-Sepharose). In the first method we used a linear KCl gradient. The asparaginase activity presented about 90% of recovery. In the second method, the elution employed either Na_2SO_4 or $MgSO_4$ decreasing linear gradient. In all the experiments the buffer used was that employed in enzyme extraction because of the enzyme stability. So far, the ion exchange chromatography showed the higher resolution and recovery. Native polyacrylamide gel electrophoresis (T = 8%) were performed with the crude enzyme preparation and the fraction isolated from anion exchange chromatography. Significative proteic complexity revealed by silver stain was observed in the sample resulting from the MonoP chromatography.

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INDIRECT ORGANOGENESIS OF *CALENDULA OFFICINALIS* L.

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Calendula Officinalis is an ornamental plant with medicinal properties, used in the pharmaceutical industry, of cosmetics and foods. The increasing interest for the phytoterapy and great demand to this plants make us think about the possible biotechnological manipulation of this species. With this objective we want to establish the protocol of organogênese of calendula. The work has been lead in the UEPG vegetal biotechnology laboratories, using cotyledonary explantes from germinated seeds *in vitro*. The medium used was MS supplemented with the respective vitamin complex, 30 g.L^{-1} sucrose, 100 mg.L^{-1} inositol, 0.5 g.L^{-1} MES, 1 mg.L^{-1} BAP and solidified with 7 g.L^{-1} agar. The treatment consisting of different hormones concentrations: Kinetin, BAP, and one mixing of these with ANA. The essay was set up in a hazardous randomized design. Percentage of explantes with buds, the number of shoots per explante, plants development, micropropagation and acclimatization essays were recorded. The great variability between and inside of assays, it makes the statistics analysis impossible but a great effect in the induction of organogenesis for BAP hormone alone or conjugated with ANA, followed for the treatment with the Kinetin and Kinetin with ANA respectively could be noticed. Inspite of the good result of hormone BAP all the regenerated shoots were vitrified, annulling in this way the good initial performance. The treatments based on Kinetin had been more efficient in the regeneration of plants than they had been successfully micropropagated in the middle MS medium without hormone. The acclimatization in the conditions tested until the moment, plants with or without leaves in saturated environment of humidity, had not been efficient. With this results we can say that the Kinetin with ANA made possible the attainment above 40% of explantes with buds and above of four shoots per explantes, that resulted in normal plants that are easily micropropagated , and it will be necessary to test acclimatization in new conditions. CNPq.

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PRODUCTION OF ASTAXANTHIN FROM *MUCOR JAVANICUS* : FRACTIONAL FACTORIAL DESIGN (2^{7-3})

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Carotenoids are lipo-soluble photopigments finding a variety of applications in the food industry. Among the carotenoids astaxanthin is now used extensively in fish farming. The biotechnological process for astaxanthin production is an alternative to the chemical synthesis. *Mucor* species have commom with many other members of the Zygomycetes that they produce carotenoids. The object of the present work was the examination of astaxanthin from *Mucor javanicus* (IFO 4570). Astaxanthin production was studied with a two-level fractional factorial design, varying the following conditions: carbon source (xylose or sucrose), initial pH (4.5 or 6.5), agitation (120 or 180 rpm), temperature (22 or 28 °C), time of cultivation (24 or 96 hours), light intensity (280 or 400 μE.m^{-2}.s^{-1}) and light colour in bioshaker (white or blue). Astaxanthin was analyzed by HPLC. The maximum astaxanthin production, 191 μg/100g mycelia, was obtained when cultures were continuously illuminated at blue light, wich was primarily responsible for photo-induced pigment production in *Mucor javanicus*. The results obtained are described here for the first time, suggesting new studies related to the of the light blue, intensity and pH. The yields reported could serve as the starting point for an experimental optimization of *Mucor javanicus* growth for large-scale astaxanthin production.

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PARTIAL PURIFICATION OF INULINASE FROM *Aspergillus niveus*

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Inulinases, the enzyme that hydrolyses β-2,1-fructofuranosidic linkages occuring in sucrose and fructans of the inulin type, have been isolated from a number of yeasts and filamentous fungi. Among then microbial inulinases offers interesting perspectives in industrial production of fructose syroup from inulin. *Aspergillus niveus* was isolated from rhizosphere of sunflower (*Helianthus annus*). The fungi was grown according to Ongen-Baysal et al. (1994), at 28°C , during 96 h, under orbital shake (200 rpm), using commercial inulin as carbon source. After cell harvesting, supernatant was filtered (Whatman 2) and tested for inulinase activity. Enzyme activity was assayed using 1% w/v inulin prepared in 0.1 M sodium acetate buffer pH 4.8. The strategy of purification utilized steps of ammonium sulphate precipitation (0-20% range), ion-exchange chromatography (DE-52, Whatman) with a stepwise NaCl gradient (0-1.0 M range) and gel filtration (Sephadex G-75). Enzyme was purified 18 times with yield of 16%. Inulinase recovery was 292% after these three purification steps.

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HIGH-LEVEL EXPRESSION OF MOUSE GROWTH HORMONE (mGH) BY PRIMARY HUMAN KERATINOCYTES USING A RETROVIRAL VECTOR

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The skin is considered a potential target for the development of gene therapy protocols for cutaneous or systemic diseases. The high proliferative capacity of cultured keratinocytes makes these epidermal cells ideal candidates for genetic manipulation.

We have utilized primary human keratinocytes transduced with an efficient retroviral vector (LXSN) encoding a mouse growth hormone gene (mGH). The main goals are the construction of an homologous *ex vivo* transfer protocol and the comparison with a similar however heterologous system based on the human growth hormone (hGH) gene.

After transfection of the vector LmGHSN into GP + E-86 ecotropic packaging cell line, the expression of mGH (10ng/mL) was detected in the culture medium by a specific radioimmonuassay. This supernatant was then used to infect the amphotropic packaging cell line GP + env Am12 and 36 clones were isolated by G418 selection. Clones presenting a secretion level ranging from 22-50ng mGH/10^6 cells.day (44 to 150ng mGH/mL) and a viral titer of 10^5 - 10^6 CFU/mL were chosen to infect the keratinocytes. The modified keratinocytes presented a stable *in vitro* secretion level up to 3µg mGH/10^6 cells.day (approximately 4µg mGH/mL), during serial propagation. These results are analogous to those previously obtained for the hGH model, which was already utilized for grafting into immunodeficient dwarf mice (little/scid).

Half-life determinations for hGH and mGH are now being carried out trying to verify a possible difference of plasma clearance rate in mice, in order to correlate those data with the levels of GH secreted *in vivo* by the genetically modified keratinocytes.

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USE OF AQUEOUS TWO-PHASE SYSTEMS ON PERFORATED ROTATING DISC CONTRACTOR FOR CONTINUOUS EXTRACTION OF *Schistosoma mansoni* RECOMBINANT ANTIGEN.

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Schistosomiasis is the second most prevalent tropical disease with more than 200 millions poeple infected and one-sixth of the world's population at risk. Sm13 is a 13 KDa tegumental antigen of *S. masoni*, potencially important for protective immunity. The aqueous two-phase systems (ATPS) have been used by the biochemical industry as alternative method of pre-chromatographic purification and used in continuous operation in large scale for separation and analysis of biological materials. ATPS presents many advantages over the methodologies usually used for separation due to high yields of product recovery, its economical cost, etc. This work describes the continuous extraction of Sm13 from a recombinant *E. coli* impure extract, the efficiency of separation in perforated rotating disc contractor (PRDC) using ATPS formed by poly(ethylene) glycol (PEG) and phophate salts. The extraction of Sm13 in the PRDC is operated in countercurrent and in a continuous mode for 70 minutes with the dispersed phase composed by PEG 3350 (19.7%) and the continuous phase composed by phosphate salts (17.7%). The parameters analysed were: rotation speed (140 and 220 RPM), dispersed phase speed (2, 3 and 4 ml/min) and pH value (6.0, 7.0 and 8.0). The best results were obtained in the systems with rotation speed of 140 RPM, dispersed phase speed of 4ml/min and pH = 7.0 at the 60 min time. It was observed that antigen partition in the ATPS is greatly affected by changing the parameters analysed.

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In vivo Monitoring of Maltose Fermentation by *Saccharomyces cerevisiae*

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The most abundant fermentable sugars in brewer's wort are maltose (~50%), maltotriose (~15%) and glucose (~10%). However, only when half of the glucose in wort has been taken up by the yeast will the uptake of maltose and maltotriose commence. The presence of glucose represses *MAL* genes, and it also promotes the inactivation of the maltose permease. The maltose permease plays a pivotal role in maltose fermentation: transport across the plasma membrane is the rate limiting step in its metabolism, and maltose uptake trough the permease is required for *MAL* gene induction. We have thus constructed an engineered yeast with the maltose permease tagged with the green fluorescent protein (GFP). The fluorescent signal from GFP can be used to monitor the expression, subcellular localization and dynamics of protein trafficking in living cells. The coding sequence of GFP was integrated in frame to the C-terminus of the permease gene by homologous recombination, and the strain obtained showed the same maltose fermentation and transport rates as the wild-type strain. This maltose permease tagged with GFP is localized at the plasma membrane, and its expression is induced by maltose and repressed by glucose. Our results also show that high concentrations of glucose stimulate the internalization of the fluorescence into the vacuole, where the permease is degraded. This strain was thus used to monitor the fermentation profile of maltose-glucose mixtures with a fluorometer and also by fluorescence microscopy. The possibilty to measure GFP fluorescence on-line and in real time allows for its use for non-invasive control and optimization of fermentation processes

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ANTIOXIDANT ACTIVITY OF GERANIIN IN HAIRY ROOTS OF *Phyllanthus tenellus* Roxb AND ITS INDUCTION BY ABIOTIC ELICITORS

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The antioxidant substances are some of the most important groups of phenolic compounds produced by plant cells. These substances play a role as oxygen free radicals scavengers avoiding their action on the human cells aging and participating in the defense response of plant cells. Studies on the *in vitro* induction and the evaluation of antioxidant activity of phenolic compounds biosynthesized by transgenic hairy roots of *Phyllanthus tenellus* are in course in our laboratory. The transgenic hairy roots Clone 1 of *P. tenellus* were induced by the infection with the strain R1601 of *Agrobacterium rhizogenes* containing the plasmid pBIN19 *gus*-INTRON. The genetic transformation was confirmed by the PCR amplification of a fragment with 477 pb from the reporter gene *nptII*. The transgenic hairy roots were submitted to $CuCl_2$ 50µM during 6, 12 and 24 hs; to cold temperature of 4°C and warmth of 37°C during 1, 6 and 12 hs and to the light irradiation during 1, 7 and 15 days. The phenolic compounds were extracted and isolated in Shimadzu HPLC with Merck column RP18 employing formic acid 10% as isocratic system. The induced substances were identified by comparison with knowledge pattern and the antioxidant activity was evaluated by the DPPH method. It was observed that the tannin geraniin with the RT 3,3 min was induced by all of the treatments. However, the influence of the different agents was heterogeneous. The higher levels of the substance was observed after 7 days of incubation under light irradiation followed by the treatment with 12 hs of incubation under cold temperature and the 6 hs of incubation with $CuCl_2$. The antioxidant activity assay showed that geraniin was a potent antioxidant presenting CI_{50} = 7,46µg/mL. The results suggested that geraniin play a role on the defense of *P. tenellus* plants which normally grow in adverse habitats. Studies are in course to identify the other phenolic substances of *P. tenellus* and their antioxidant activity. Supported by FAPERJ and FENORTE.

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CLONING AND EXPRESSION OF HYBRIDS AND TRUNCATED FORMS OF PNEUMOCOCCAL SURFACE PROTEIN A (PSPA) OF *Streptococcus pneumoniae*.

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Streptococcus pneumoniae is a pathogenic bacteria of great interest in public health once it causes diseases as pneumonia and meningitis. The currently available pneumococcal vaccine consists of capsular polisaccharide (PS) of 23 different strains, which makes it very expensive. Moreover, PS vaccines induces thymus-independent immuno-response which is not effective in children. The use of protein antigens could broaden vaccine coverage, reduce costs and be effective in children. The pneumococcal surface protein A, PspA, has been shown to be immunogenic and protective in experimental animals. It is a virulence factor that inhibits the activation of complement system avoiding fagocitosis of the bacteria. PspA can be divided on 3 families and 6 clades based on its primary sequence. The serological difference related to its genetic variability suggests the inclusion of PspAs of families 1 and 2 in order to have a broad protection spectra. The present work proposes the obtention of hybrids containing imunogenic epitopes of members of the families 1 and 2 to the inclusion in a vaccine against *Streptococcus pneumoniae* and truncated forms of the protein in order to study the contribution of specific epitopes in its imunogenicity. The pspA genes of the family 2 (clade 1 and 2) and of family 2 (clade 3 and 4) were isolated from genomic DNA of *S. pneumoniae* isolates (by Dr. Eliane Miyaji) and cloned initially in the eucariotic vector pTARGET. The hybrids between families 1 and 2 were planned to contain the main immunogenic epitopes linked by a prolin rich region of the protein which has the function of wild protein of link a colin tail (attached to cell suface) to the α-helix exposed region. The gene fragments were amplified and linked in the expression vector pAE-6xHis between Xho and Kpn I restriction sites. The expression of protein will be carried out in *E. coli* BL21-DE cells and purification should be performed in an Akta Prmime apparatus (Amersham Biosciences) by Ni^{++} chelating and anion-exchange chromatographies.

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CLONING AND EXPRESSION OF TETANUS TOXIN FRAGMENT C IN FUSION WIT THE PERTUSSIS TOXIN S1 SUBUNIT

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The aim of this work is to develop a bivalent vaccine against tetanus ar pertussis. We used the highly immunogenic tetanus toxin fragment C in fusion with t truncated version of the pertussis toxin subunit S1(Pizza et al., Science 246, 497, 198 The cDNAs coding to mutated S1subunit and fragment C were cloned into the pRSET (S1 at C-terminal) and into pET 32a (S1at N-terminal) vectors which provide a high-lev expression of heterologous proteins under control of the T7 promoter. All vect constructions were confirmed by DNA sequencing and restriction analysis. These clon were used to transform *E. coli* BL21 (DE3) where the *lacUV5* promoter directs t transcription of T7 RNA polymerase gene, which is inducible by IPTG. The bacter lysates from induced cultures were analyzed through a 10% SDS-PAGE. The expecte fusion proteins bands were present with ~ 75 kDa consisting of 50 kDa fragment C a ~ 25 kDa of S1 subunit, from the transformants with the construction pRSETA-FC/ and the a band of ~ 90 kDa consisting of 50kDa fragment C, ~ 25 kDa S1 plus ~15 kD of thioredoxin from the transformants with the construction pET32A-S1/FC. The proteins were purified through Ni^{+2}-charged chromatography. Western blot analysis the purified fusion proteins showed that they are both recognized by polyclonal ar tetanus toxoid and anti-pertussis toxin sera. These results clearly show that recombina S1 expressed in the construct at the amino-terminal fusion to the tetanus toxin fragme C, reacted with high specificity with anti-pertussis antibodies. ELISA and vaccination animal model with these proteins are currently under investigation.

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ESTUDO DAS CONDIÇÕES DE PRODUÇÃO DE PROTEASES POR *Aspergillus sydowii*

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Objetivo: Este trabalho tem o objetivo o estudo das condições de produção de proteases extracelulares produzidas *Aspergillus sydowii* em diferentes meios de culturas, temperaturas e velocidades de agitação.
Metodologia: O microorganismo *Aspergillus sydowii*, isolado de peixe da região do Nordeste brasileiro, foi cultivado em meio de cultura com diferentes fontes de nitrogênio,- farinha de soja, farinha de aveia ou farinha de arroz (1% p/v), incubados em agitador orbital nas velocidades de 180 e 200 rpm em duas diferentes temperaturas (28° e 30°C). Os ensaios foram realizados em frascos de Erlenmeyers contendo os diferentes meios, inoculados de modo a obter uma concentração final de 10^5 esporos/ml, em agitador orbital nas condições de temperatura e agitação estabelecidas para cada experimento, durante 96 horas. A cada 24 horas foram retiradas alíquotas para determinação proteína total, atividade proteásica e pH. Para determinação do conteúdo protéico utilizada a metodologia de Bradford (1976) e a atividade proteásica foi determinada segundo o método de Ginther (1979) que utiliza azocaseína 1% (p/v) como substrato.
Resultados: Ensaios realizados a velocidade de 180 rpm com a farinha de aveia nas temperaturas de 28 e 30°C, obtivemos 118 e 210 U/mg no tempos de crescimento de 96 e 48 horas, respectivamente. E para os meios de soja e arroz atividade proteásica específica foi menor. Enquanto que experimentos realizados a 200 rpm a melhor atividade específica obtida foi com o meio que continha soja, obtendo 1024 U/mg em 96 horas de crescimento a 30°C, seguida do meio de arroz com 1000 U/mg em 96 horas.
Conclusão: A atividade proteásica específica foi influenciada pela velocidade de agitação, os resultados obtidos com a maior velocidade foram superiores .