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**Determinação dos parâmetros cinéticos de
resistência térmica da Proteína Verde Fluorescente
recombinante (*GFPuv*)**

Marina Ishii

Dissertação para obtenção do título de
MESTRE

Orientadora:
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Verde Fluorescente Recombinante (*GFPuv*)**

**Comissão Julgadora
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Dissertação para obtenção do título de Mestre**

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para minha formação.*

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pelo incentivo e amor sempre presentes.*

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Lista de Abreviaturas e Símbolos

β - can	beta can
λ	comprimento de onda
$\mu\text{g/mL}$	microgramas /mililitro
$^{\circ}\text{C}$	graus <i>Celsius</i> -
$\text{\AA}$	ângstron
Aa	atividade de água
Ala	alanina
$\text{C}\alpha$	carbono alfa
$\text{C}\beta$	carbono beta
Ca^{2+}	ion cálcio
cm	centímetro
Ea	Energia de Ativação
EDTA	ácido etilenodiaminotetracético
g	gramas
GFP	proteína verde fluorescente (<i>green fluorescent protein</i>)
GFPuv	proteína verde fluorescente recombinante
Gly	glicina
HIC	<i>Hydrophobic Interaction Column</i>
IB	indicador biológico
K	graus Kelvin
k	taxa de inativação constante
kcal	kilocalorias
KDa	kilodalton
$\log_{10}$	logarítmo decimal
Met	metionina
Mg^{2+}	íon magnésio
mg/mL	miligramas /mililitro
min	minutos
mM	milimolar
nm	nanômetro

pH	potencial hidrogeniônico
Phe	fenilalanina
PME	pectinesterase
R²	coeficiente de correlação
s	segundos
SAL	Sterility Assurance Level
Ser	serina
Thr	treonina
TPP	three phases partitioning
Tyr	tirosina
UHP	ultra high pressure
UV	ultra-violeta
Val	valina

RESUMO

Células transformadas de *E.coli DH5-α* expressando a proteína verde fluorescente (*GFPuv*, pico de excitação e emissão de 394nm e 509nm) foram submetidas a extração pelo método de partição de três fases (TPP) e o extrato obtido purificado por cromatografia de interação hidrofóbica (HIC).

O objetivo principal deste trabalho foi estudar a termoestabilidade da *GFPuv* extraída, para avaliar a sua possível utilização como indicador biológico econômico, de resposta rápida e precisa para processos térmicos de esterilização utilizando o calor úmido.

A estabilidade térmica da proteína foi estudada em diferentes soluções-tampão (acetato, fosfato e tris-HCl 10mM) no intervalo de valor de pH de 5,0 a 9,0 e, em temperaturas entre 75° e 95°C. Os parâmetros de resistência térmica determinados foram: o tempo de redução decimal (Valor D - min), valor z (°C), coeficiente Q_{10} e valor de energia de ativação (kcal/mol).

A termoestabilidade da *GFPuv*, expressa em valor D, mostrou correlação linear para valores de pH $\geq 5,50$, em tampão acetato. Em tampão fosfato, para valores de pH $\geq 7,50$ a estabilidade térmica da proteína foi independente do valor de pH da solução. Em tampão tris-HCl, o valor D mostrou-se inconstante ao aumento do valor de pH da solução.

No intervalo de temperatura estudada, em tampão acetato a *GFPuv* apresentou melhor termoestabilidade (E_a de 19,27 kcal/mol) do que em tampão fosfato (E_a de 26,18 kcal/mol ao valor de pH 6,5) e em tampão tris-HCl (E_a 28,19 kcal/mol ao valor de pH 7,0).

Em tampão acetato e tris-HCl ao valor de pH 7,0, a termoestabilidade da proteína mostrou-se equivalente. Entretanto, em tampão fosfato aos valores de pH 7,5 e 8,0 e em tampão tris-HCl aos valores de pH 8,0 e 8,5 a *GFPuv* apresentou menor estabilidade térmica.

A *GFPuv* apresenta potencialidade para ser utilizada como indicador biológico em processos térmicos que utilizam calor úmido às temperaturas inferiores a 100°C.

ABSTRACT

Transformed cells of *Escherichia coli* DH5- α expressing recombinant green fluorescent protein (*GFPuv*, excitation and emission peaks at 394nm and 509nm), were subjected to the three-phase partitioning (TPP) method and the release extracts were eluted through methyl HIC column with a buffer solution (10 mM Tris-HCl, 10mM EDTA, pH=8.0). The purpose of this work was to study the thermal stability of the TPP-extracted recombinant protein, *GFPuv*, to determine its utility as a quick, accurate and economical biological indicator for moist heat-treatments.

The thermal stability of the extracted *GFPuv* was studied in different buffer solutions (acetate, phosphate and tris-HCl 10mM) in the range of pH between 5.0 and 9.0 and at temperature between 75–95°C. The thermal resistance parameters determined were: decimal reduction times (D-values, min), z-value (°C), Q_{10} coefficient and Activation Energy (Ea, Kcal/mol).

The thermal stability of *GFPuv*, expressed in D-values, showed linear correlation for pH ≥ 5.50 in acetate buffer. In phosphate buffer, for pH ≥ 7.50 the thermal stability was independent of pH value. In tris-HCl buffer the D-value was shown variable with the increase of pH value.

In the studied temperature range, the acetate buffer at pH 6.0 presented better thermal stability for *GFPuv* (Ea 19.27kcal/mol) than phosphate (Ea 26.18 kcal/mol at pH 6.5) and tris-HCl buffer (Ea 28.19 kcal/mol at pH 7.0).

In acetate and tris-HCl buffers at pH 7.0, *GFPuv* showed equivalent thermal stability. However, *GFPuv* showed lower thermal stability in phosphate buffer at pH 7.5 and 8.0 and in tris-HCl buffer at pH 8.0 and 8.5.

The TPP-extracted *GFPuv* has great potential to be applied as a biological indicator in moist heat processes at temperatures below 100°C.

JUSTIFICATIVA

O desenvolvimento de pesquisas para a melhoria das condições de desinfecção e de esterilização, é de suma importância para prevenir a disseminação de contaminações e infecções em ambientes da saúde.

A confirmação do teste de esterilidade de produtos, de materiais e equipamentos médico-hospitalares depende da constatação de indicadores biológicos (IBs) sobreviventes, análise esta que demanda de 2 a 10 dias, ocasionando a demora da liberação de produtos para o consumidor e restringindo a rotatividade de itens hospitalares.

Devido a importância da validação dos processos de esterilização em ambientes pelo calor úmido torna-se necessária a utilização de um indicador biológico que apresente resultados rápidos e precisos sobre a eficiência do processo.

A utilização da microscopia de fluorescência na observação da expressão da proteína verde fluorescente (*GFPuv*) por células germinantes originárias de esporos sobreviventes aos tratamentos de desinfecção e de esterilização constituir-se-á em um método rápido e preciso em substituição ao procedimento convencional de incubação do bioindicador por 24-48h para verificação de sobreviventes. A detecção destes em quatro horas por constatação da presença da *GFPuv*, deverá ser otimizada por espectrofotometria de fluorescência ou por microscopia de fluorescência.

Pretendeu-se expressar a *GFPuv* em células competentes de *Escherichia coli* DH5- α como fonte da proteína a ser isolada e suas propriedades físicas estudadas como potencial bioindicador de desinfecção. Avaliou-se a cinética de crescimento de *E. coli* e, concomitantemente, a produção de *GFPuv*.

Estudou-se o melhor método para a extração da proteína do meio celular, e, avaliou-se a cinética de destruição térmica da *GFPuv*, através da obtenção do valor D, do valor z e da energia de ativação térmica (E_a), em relação à extinção da *GFPuv*.

O objetivo principal deste projeto foi o estudo da termoestabilidade da *GFPuv* em diferentes composições de soluções-tampão, e valores de pH, avaliando a possível utilização da *GFPuv* como indicador biológico em processos de esterilização à temperaturas inferiores a 100°C.

REVISÃO BIBLIOGRÁFICA

1. Proteína Verde Fluorescente (GFP) da *Aequorea victoria*

A bioluminescência é um fenômeno natural observado em diversas espécies de organismos marinhos e terrestres. No ambiente terrestre, o vaga-lume é o representante mais conhecido.

No oceano, estas espécies aparecem nos diferentes grupos de microrganismos marinhos, compreendendo desde o zooplâncton (protozoários dinoflagelados) como *Noctilucas*; pequenos hidrozoários como a *Obelia geniculata*; medusas como *Aequorea victoria*; ctenóforos como as *Deiopeas* e o *Beroe forshalii*; crustáceos como o camarão *Euphasia pacifica*; e peixes demersais como *Porichthys porosissimus*. Estes animais marinhos utilizam-se da bioluminescência para afastar predadores, para camuflagem mimetizando outras espécies, para atrair suas presas, para atrair seus parceiros no período reprodutivo e como fonte alternativa de energia (BARNES, 1991).

O mecanismo químico de produção de luz por organismos marinhos difere para cada espécie. A maioria apresenta somente quimiluminescência ou bioluminescência que resulta de uma reação química mediada por fotoproteínas.

O exemplo mais bem elucidado é a reação de oxidação da luciferina a oxiluciferina catalisada pela enzima luciferase, produzindo luz. Alguns mecanismos químicos de bioluminescência mediados por luciferinas, são apresentadas na figura 01.

Outros mecanismos de luminescência são independentes de substrato e apresentam sistemas próprios. A fluorescência ocorre quando um elétron excitado é deslocado para uma órbita de maior energia. Ao retornar para o estado mais estável ou de menor energia, o elétron emite um “fóton”.

Tabela 01. Exemplos de organismos que apresentam bioluminescência (Chalfie & Kain, 1998)

Tipo de organismo	Gênero representativo	Mecanismos/ Componentes de emissão de fluorescência	Característica/Função
Bactéria	<i>Photobacterium</i>	Flavina reduzida e aldeído da cadeia longa de luciferase	emissão constante
Dinoflagelados	<i>Noctiluca</i>	Cintilação através de organelas celulares ($\lambda=470\text{nm}$)	Brilho em flashes rápidos para afugentar predadores
cnidários medusas hidróides	<i>Aequorea</i> <i>Obelia</i>	Ca^{2+} , coelenterazina / aequorina núcleos imidazólicos-pirazínicos ($\lambda=460 - 510\text{nm}$), emissão: GFP	brilho em flashes rápidos para afugentar predadores. Mais estudado.
ctenóforos	<i>Beroe</i>	Ca^{2+} , coelenterazina ($\lambda=460\text{nm}$)	brilho em flashes rápidos para afugentar predadores
crustáceos	<i>Cypridina</i>	núcleos imidazólicos-pirazínicos ($\lambda=465\text{nm}$)	liberar enzimas em substratos
insetos vaga-lume	<i>Photinus</i> , <i>Photuris</i>	núcleo benzotiazólico, ATP, Mg^{2+} ($\lambda=550-580\text{nm}$)	Flashes, padrão específico de cinética; afastar predadores, acasalamento
Tunicados	<i>Pyrosoma</i>	organelas celulares envolvendo bactérias ($\lambda=480-500\text{nm}$)	fileira brilhante de função desconhecida
Peixes	<i>Porichthys</i>	autoluminosidade dependente da luciferina ($\lambda=485\text{nm}$)	Fotóforos ventrais, camuflagem, corte

Figura 01. Mecanismo químico de bioluminescência de quatro diferentes luciferinas (Chalfie & Kain, 1998).

2. Bioluminescência da *Aequorea victoria*

O mecanismo químico de produção de bioluminescência da *Aequorea victoria* vem sendo estudado há trinta anos por CHALFIE AND KAIN, 1998.

A *Aequorea victoria* é um cnidário de corpo totalmente transparente em forma de guarda-chuva, com diâmetro entre 7 e 10 cm que apresenta grânulos bioluminescentes ao redor de seu corpo, formando um círculo.

Figura 02. *Aequorea victoria*

(<http://www.mbari.org/~kraskoff/MBARI/aequorea.htm>)

Em 1961, foi observado que o extrato destes grânulos apresentava luminescência azul (chamaram de extrato de aequorina), diferentemente da bioluminescência observada no organismo vivo.

Experimentos realizados com a medusa *Aequorea victoria* mostraram que quando o animal era imerso em uma solução de cloreto de potássio emitia uma luz verde e quando mergulhada em água destilada emitia luz verde mais tênue que aos poucos se transformava em azul, provavelmente devido à lise celular.

Argumentou-se que se tratava de dois tipos diferentes de substâncias luminescentes: a aequorina, uma fotoproteína dependente de Ca^{2+} , que produz luz azul apresentando um largo espectro de emissão em $\lambda = 460\text{nm}$ e a proteína verde fluorescente (GFP) que apresenta pico de emissão verde em $\lambda = 488\text{nm}$ quando excitada por luz ultravioleta. Trata-se de um sistema

acoplado, onde a luz azul emitida pela fotoproteína aequorina dependente de cálcio é transferida para a proteína verde fluorescente (*GFP*), que emite luz verde ao ser excitada diretamente por radiação exógena (WARD, *et al*, 1980).

Biologicamente, a *GFP* atua na mudança da cor da bioluminescência de azul para verde nos celenterados luminosos e para intensificar o fóton de emissão de luz. Todos os celenterados utilizam a mesma luciferina nas reações bioluminescentes, produzindo uma proteína ligada, a oxiluciferina que emite luz azul na ausência da *GFP*. Entretanto, na presença da *GFP*, a luz emitida é verde, sendo idêntica quanto às propriedades espectrais de emissão fluorescente do espectro da *GFP* quando excitada diretamente por radiação exógena.

3. Propriedades Gerais da Proteína Verde Fluorescente selvagem (*GFP*) e recombinante (*GFPuv*).

3.1. Estrutura

A *GFP* da *A. victoria* ou *GFP* selvagem é um polipeptídeo que contém na sua estrutura primária 238 aminoácidos, sendo um monômero de massa molar de 27 kDa (CODY *et al*, 1993).

Segundo YANG *et al* (1996), a estrutura cristalina da *GFPuv* é o conjunto de dois monômeros dispostos em 11 fitas formados por β -folha pregueada externamente em forma de barril ou cilindro, o qual abriga em seu centro geométrico o grupo fluoróforo que permanece ligado ao cilindro por α -hélices. A estrutura tridimensional em forma de cilindro apresenta aproximadamente um diâmetro de 30 Å por 40 Å de altura (figura 03). Um mapeamento de densidade eletrônica revelou tratar-se de um dímero. Este tipo de estrutura quaternária proteica, não relatada anteriormente, foi denominada de β -can. O grupo fluoróforo da *GFP* selvagem é formado por três aminoácidos: Serina, Tirosina e Glicina (Ser⁶⁵-Tyr⁶⁶-Gly⁶⁷) (figura 04).

Figura 03. Estrutura barril β -can da *GFPuv*.

(http://www.gla.ac.uk/centres/cellengineering/Fluo_Labs_Support.htm)

Figura 04: Reação de cicização autocatalítica e oxidação para formação do grupo fluoróforo. (<http://www.biochem.mcw.edu>).

Segundo os autores HEIM *et al.* (1995), a *GFPuv* nascente não apresentava fluorescência, e a formação do grupo fluoróforo ocorria somente após algumas horas. O grupo fluoróforo forma-se através de duas reações: uma reação de cicização autocatalítica entre a carbonila da Tyr⁶⁶ e o grupo amino da Gly⁶⁷, e da carbonila da Ser⁶⁵ e o grupo amino da Tyr⁶⁶ formando uma ligação covalente e uma etapa lenta dependente de oxigênio

onde a ligação simples entre os carbonos $C\alpha$ - $C\beta$ da Tyr⁶⁶, é oxidada à dupla ligação (figura 04).

O resultado é a formação de duplas conjugadas nesta estrutura que tem propriedades fluorescentes.

Dentre as variantes da *GFPuv* utilizadas como ferramenta de estudo da expressão gênica, está a que emite fluorescência verde 18 vezes mais intensa que a *GFP* selvagem. Tem sido estudada em células de procariotos (CRAMERI *et al.*, 1996).

3.2. Absorbância e emissão de luz

A *GFP* selvagem absorve UV e luz azul, com um pico máximo de absorbância a 395 nm e um menor pico a 470 nm, e emite luz verde com um pico máximo de emissão a 509 nm e um pequeno espalhamento em 540 nm. Esta *GFP* quando inserida em procariontes precipita no citoplasma tornando-se não fluorescente (WARD *et al.*, 1980).

A proteína verde fluorescente recombinante (*GFPuv*) emite máxima fluorescência quando excitada por luz ultravioleta (360–400nm). Esta *GFPuv* é muito utilizada em estudos de expressão gênica porque pode ser facilmente visualizada sob luz UV. A *GFPuv* foi recombinada através de técnicas sofisticadas de mutações puntiformes no DNA da *GFPuv in vitro* (CRAMERI, *et al.*, 1996). O resultado foi a substituição de três aminoácidos (Phe⁹⁹ por Ser, Met¹⁵³ por Thr e Val¹⁶³ por Ala) da *GFP* selvagem, resultando na *GFPuv*. A *GFPuv* apresenta fluorescência 18 vezes mais intensa do que a *GFP* selvagem quando expressas por *E. coli*. Geralmente, mutações interferem nos picos de absorção e emissão das variantes da *GFPuv*. Porém, a *GFPuv* conserva esta propriedade mantendo o comprimento de onda de emissão em 509nm (CRAMERI, *et al.*, 1996).

Tabela 02. Propriedades físicas da GFP selvagem

Massa Molar do monômero	27kDa
Ponto Isoelétrico	4,6 – 5,1
Emissão máxima de fluorescência	509nm
Rendimento quântico da fluorescência	0,80
Proporção de absorção 397nm/380nm	1,25

3.3. Solubilidade da Proteína Verde Fluorescente

Segundo CRAMERI, *et al.*, (1996), a GFP selvagem expressa em *E. coli* apresentou-se insolúvel, pouco fluorescente e precipitou no citoplasma. A GFPuv expressa nas mesmas condições, apresentou-se solúvel e com fluorescência intensa, porém mais citotóxica e com maior possibilidade de dimerização. O tempo de formação do fluoróforo mostrou-se de 2 a 3 vezes mais lento quando comparado ao tempo de formação do fluoróforo da GFP selvagem expressa pelo mesmo microrganismo.

3.4. Estabilidade da proteína (*in vitro*)

A GFP selvagem é resistente a temperaturas de até 70°C, a pH alcalino (inferior a 12), detergentes, solventes orgânicos e algumas proteases (ROBART & WARD, 1990). Alguma fluorescência é observada quando nanogramas da proteína nativa é aplicada em 1% de dodecil sulfato de sódio (SDS) em gel de poliacrilamida (INOUE & TSUJI, 1994).

3.5. Dimerização

A GFPuv é fluorescente em ambas formas, tanto no monômero quanto no dímero. A proporção das formas monoméricas ou diméricas depende da concentração da proteína no meio. A GFP selvagem dimeriza via interações hidrofóbicas em concentrações de proteína de 5 a 10 mg/mL.

A dimerização resulta na diminuição da absorção a 470nm e em concomitante aumento da absorção em 395nm. A *GFPuv* tem grande probabilidade em apresentar-se na forma dimerizada (CUTLER & WARD, 1997).

4. Expressão gênica da Proteína verde fluorescente e variantes.

4.1. Aplicações

A *GFPuv* tem sido nos últimos anos uma ferramenta básica para estudos de biologia molecular, biotecnologia, citometria, bioquímica, biofísica e genética.

Isto é possível pois a *GFPuv* é espécie independente e pode ser inserida em diversos hospedeiros de espécies diferentes. A *GFPuv* pode ser expressa com sucesso em bactérias (CHALFIE *et al.*, 1994), leveduras (KAHANA *et al.*, 1995), plantas, drosófilas (WANG & HAZELRIGG, 1994), peixes (AMSTERDAM *et al.*, 1996) e em células de mamíferos.

4.2. Clonagem da Proteína Verde Fluorescente com outras proteínas.

O gene que expressa a *GFPuv* pode ser clonado com um gene que expressa outra proteína. Esta clonagem é possível pois em muitos casos as proteínas apresentam semelhanças estruturais com N- ou C-terminal as quais possibilitam a ligação entre os genes, mantendo sua expressão e a atividade de ambas proteínas (STEARNS, 1995).

As aplicações destas clonagens são incontáveis.

WANG & HAZELRIGG (1994), relataram que *GFP* selvagem clonada com proteínas utilizadas em ensaios imunológicos, pode aumentar a sensibilidade e a resolução das colorações utilizadas em técnicas padrões para anticorpos e também eliminar as etapas de fixação, permeabilização celular e incubação do anticorpo se for utilizado um anticorpo fluorescente.

A *GFPuv* clonada permite estudos da cinética molecular em células vivas, localização de proteínas utilizando a *GFPuv* como marcador luminoso (FLACH *et al.*, 1994).

WEBB *et al.* (1995) têm feito estudos da formação do endósporo em *Bacillus subtilis*, fundindo *GFPuv* com a proteína CotE. Estudo semelhante foi realizado por LEWIS & ERRINGTON, (1996), que fundiram *GFPuv* com a proteína DacF e SpoIVA do *B. subtilis* para detectar o gene de expressão célula-específica e sua localização subcelular durante a esporulação.

FLACH *et al.* (1994) visualizaram o movimento de ida e volta do RNA transportador entre o núcleo e o citoplasma utilizando a proteína histona fluorescente no núcleo de leveduras.

KAETHER & GERDES (1995) visualizaram o transporte de proteínas ao longo de membranas secretoras utilizando a proteína verde fluorescente.

SHIGA *et al.* (1996) estudaram a morfogênese das células de drosófilas utilizando como ferramenta uma β -galactosidase fluorescente nuclear.

HEINLEIN *et al.* (1995) relataram a interação do tobamovírus com o citoesqueleto do pé-de-tabaco através da fluorescência dos microfilamentos.

Em células humanas SULLIVAN *et al.* (1995) observaram a seqüência *in vivo* de formação do centrômero no DNA humano marcado com EGFP (Enhanced GFP).

A proteína verde fluorescente e suas variantes são utilizadas como marcadores intracelulares, podendo ser facilmente visualizadas.

4.3. Aplicações gerais

A *GFPuv* pode ser utilizada para diversas finalidades, dentre elas: (i) diferenciar culturas mistas; (ii) monitorar a expressão gênica frente a múltiplos promotores em células, tecidos e organismos; (iii) observar a permeabilização da membrana; (iv) localizar e/ou analisar em tempo real interação proteína-proteína; (v) visualizar o transporte e a função de organelas intracelulares e também a estrutura do citoesqueleto e (vi) separar

culturas mistas em células que expressam diferentes variantes de GFP utilizando FACS “cell sorter” (CHALFIE & KAIN, 1998).

4.4. Usos em procariontes

A GFP da *A. victoria* é a única ferramenta que permite o monitoramento da expressão gênica e a localização intracelular de proteínas em células vivas.

A GFPuv não requer cofatores, catalisadores ou substratos para sua atividade (CHALFIE *et al.*, 1994). Pode ser expressa com funcionalidade em diversas espécies bacterianas.

A temperatura de incubação do microrganismo pode afetar a fluorescência.

A formação do grupo fluoróforo parece ocorrer no terço final da fase logarítmica do crescimento bacteriano. A formação do fluoróforo ocorre aproximadamente duas horas após a síntese proteica.

5. *Escherichia coli* DH 5- α .

A *E. coli* foi o veículo utilizado para a produção da proteína verde fluorescente (GFPuv) por ser um dos microrganismos mais estudados até hoje. Tem seu mapa cromossômico definido e bem caracterizado.

As células hospedeiras de *E. coli* DH5- α podem tornar-se competentes facilmente, isto é, apresentar permeabilidade a plasmídeos em determinadas condições. Muitos autores utilizaram a *Escherichia coli* como célula hospedeira para produção de mutantes da proteína verde fluorescente (HEIM *et al.*, 1996).

A *Escherichia coli* é sensível a temperaturas superiores a 45°C, porém, a GFP selvagem apresenta fluorescência mesmo quando exposta à temperaturas de 70°C.

A *GFPuv* apresenta uma grande vantagem como indicador biológico em relação ao método de quantificação. A análise quantitativa da fluorescência remanescente das amostras submetidas aos processos de desinfecção pode ser determinada por espectrofotometria de fluorescência, tornando o método rápido e preciso. A determinação qualitativa pode ser determinada por microscopia ultravioleta.

Conhecendo-se as propriedades físico-químicas da *GFPuv* e acreditando serem viáveis como indicador biológico, será de grande valia a transformação de cepas de *E. coli* que apresentem fluorescência.

6. Processos de Esterilização

Esterilização é o processo que objetiva destruir todas as formas de vida com capacidade de desenvolvimento durante os estágios de conservação e de utilização de produtos. Conservar é manter as características do produto durante a sua vida útil de armazenamento (vida de prateleira) a temperatura ambiente.

Esterilidade ou nível de segurança é a incapacidade de desenvolvimento das formas sobreviventes ao processo de esterilização, durante a conservação e utilização do produto. A manutenção do nível de esterilidade conferido a um produto garante o prolongamento da vida útil e depende das condições operacionais de pré-esterilização, de esterilização e pós-esterilização.

Os métodos de esterilização permitem assegurar níveis de esterilidade compatíveis às características exigidas em produtos farmacêuticos, médico-hospitalares e alimentícios. O método escolhido depende da natureza e da carga microbiana inicialmente presente na matéria-prima considerada. O calor, a filtração, a radiação e o óxido de etileno são agentes esterilizantes (VESSONI PENNA *et al.*, 1997).

6.1. Esterilização por calor úmido

De todos os métodos de esterilização, o calor úmido na forma de vapor saturado sob pressão é o mais freqüentemente utilizado e mais confiável. Não tóxico, relativamente barato e esporocida. O vapor aquece rapidamente o material e penetra em sua estrutura. Este método não pode ser utilizado em materiais ou produtos termossensíveis ou higroscópicos.

O princípio básico de esterilização a vapor quando realizado em autoclave é o tratamento de cada item com vapor a temperatura e pressão adequados por um tempo específico, com a finalidade de destruir os microrganismos por denaturação irreversível de suas enzimas e estruturas proteicas.

São quatro os parâmetros para esterilização a vapor: temperatura, pressão, tempo e concentração de vapor. O vapor ideal para esterilização é 100% saturado, com água não saturada na forma de uma fina névoa. A produção de pressão a altas temperaturas é fundamental para exterminar os microrganismos rapidamente. A temperatura específica deve ser obtida para garantir a atividade microbicida. Normalmente utilizam-se temperaturas de esterilização de 121°C por 30 minutos e 132°C por 4 minutos em autoclave com pulsos de vácuo. Esporos de *B. subtilis* e *B. stearothermophilus* são utilizados para monitorar a eficiência da esterilização (VESSONI PENNA *et al.*, 1997).

7. Importância da validação de processos de esterilização e sanitização por um indicador biológico.

O indicador biológico (IB) é uma ferramenta de fácil manipulação que permite a monitoração e validação dos processos de esterilização e desinfecção.

A otimização nos processos de desinfecção e esterilização depende da correta e criteriosa escolha, aplicação e observação das características

peculiares de cada agente físico e químico, assim como dos fatores interferentes.

Esporos de *Bacillus stearothermophilus* são considerados convenientes indicadores biológicos (IBs) na esterilização por calor úmido, à temperatura de referência de 121°C. Esporos de *Bacillus subtilis* são empregados nos processos de esterilização pelo calor seco e pelo óxido de etileno.

O número de microorganismos sobreviventes e/ou a concentração remanescente de proteína ao final do processo de esterilização, representa o efeito do processo. A destruição térmica de uma população/concentração proteica homogênea pode ser representada por um modelo de curva semi-logarítmica de sobreviventes em relação ao tempo de exposição ao agente esterilizante.

A resistência específica a um certo agente esterilizante é dada pelo Tempo de Redução Decimal (Valor D), a uma temperatura constante de referência. O Valor D é definido como o intervalo de tempo necessário, à temperatura constante para redução de 90% da população microbiana e/ou concentração proteica, inicialmente presente no produto e/ou início do tratamento.

Na escolha do indicador biológico deve-se conhecer a população inicial de esporos e/ou concentração proteica inicial e também, o Valor D requerido a uma temperatura constante. No método *overkill*, o efeito do tempo de aquecimento é avaliado em termos do número de ciclos reduzidos em uma população inicial de esporos. O nível de segurança de esterilidade (SAL – Sterility Assurance Level) depende das características do indicador biológico (número inicial de esporos e Valor D), temperatura e tempo total de exposição ao agente esterilizante (VESSONI PENNA *et al.*, 1997).

8. Características de um Indicador biológico

O indicador biológico (IB) é pode ser caracterizado por uma suspensão de microrganismos específicos ou até mesmo solução de proteínas resistentes a determinado processo térmico.

Os indicadores biológicos não devem ser patogênicos e, em caso de microrganismos, devem permitir a reprodutibilidade e a precisão do ensaio e multiplicar-se rapidamente facilitando a contagem por semeadura de profundidade (VESSONI PENNA *et. al.*, 2000).

Os indicadores biológicos devem apresentar resistência ao agente desinfetante e/ou esterilizante superior à resistência dos microrganismos originalmente presentes no produto a ser esterilizado.

Indicadores biológicos são utilizados como instrumentos para validar processos físicos e químicos de esterilização e desinfecção.

Na esterilização a vapor saturado, aplicada a produtos termoestáveis, os esporos de *Bacillus stearothermophilus* são amplamente recomendados (FARMACOPÉIA BRASILEIRA IV; USP XXIV e XXV). Além disso, os esporos são empregados para monitorar a desinfecção de aparelhos dentários e médicos, através (i) do gás plasma de peróxido de hidrogênio (ALFA, 1996), (ii) de solução de peróxido de hidrogênio alternada à solução de acetona, (iii) do vapor do formaldeído, do glutaraldeído, do hipoclorito de sódio e de compostos quaternários de amônio usados nas misturas germicidas. Esporos de *Bacillus stearothermophilus* também podem ser utilizados para classificar diferentes agentes químicos esterilizantes de acordo com o espectro de atividade.

A resistência térmica de *Bacillus stearothermophilus* é influenciada por vários fatores ambientais como a temperatura de incubação, pH e composição do meio de esporulação. (VESSONI PENNA *et. al.*, 2000).

Indicadores biológicos de *B. stearothermophilus* inoculados em fitas de papel de filtro são primariamente utilizados para determinar a eficácia de processos de esterilização pelo calor úmido. Esporos de *B. stearothermophilus* e *B. subtilis* são utilizados comercialmente em fitas de

papel, para monitorar processos de esterilização pelo vapor, calor seco ou óxido de etileno. A suspensão dos esporos em ampolas é utilizada para avaliar a esterilização de fluidos Newtonianos. Bioindicadores são ferramentas utilizadas para assegurar a esterilidade de um produto específico, por monitoramento dos ciclos de esterilização, validando o processo.

9. Uso de Proteínas como Indicadores Biológicos para Processos de Esterilização a baixas temperaturas.

O tratamento térmico de produtos por calor úmido (em temperatura $\leq 100^{\circ}\text{C}$) à pressão ambiente, é uma das principais técnicas das indústrias alimentícias e farmacêuticas para a conservação de seus produtos. O tratamento inibe a atividade enzimática e bacteriana, visando evitar alterações nos alimentos de origem orgânica, enzimática, física e química. Várias combinações de tempo/temperatura podem ser aplicadas dependendo das propriedades dos produtos e do tempo de vida de conservação desejável.

O branqueamento (*blanching*) ou escaldado é um processo térmico que consiste na imersão do material em água quente ou exposição ao vapor d'água, à temperatura entre 70°C e 100°C . O principal objetivo do branqueamento de frutas e vegetais é a ativação e/ou inativação de enzimas e retardamento da ação de bactérias contidas em frutas e hortaliças, antes de serem submetidas ao congelamento, ocasionando também a remoção de ar do interior dos tecidos, a destruição parcial dos microorganismos e a manutenção da cor e da textura dos alimentos (GAVA, 1984).

O branqueamento desenvolve as seguintes ações (EVANGELISTA, 1987):

- limpa a superfície de alimentos, reduzindo o número de microorganismos;

- amolece e entumece os tecidos vegetais, dando uma massa mais uniforme ao alimento, dentro do recipiente determinado;
- produz a inativação de enzimas que afetam as qualidades dos produtos durante e depois do processamento, como por exemplo, a inativação de fenoxidases, impedindo a despigmentação de tomates e maçãs;
- favorece a fixação da coloração de certos pigmentos de vegetais;

São exemplos de alimentos que sofrem branqueamento: hortaliças e milho na espiga no pré-congelamento; tomates que racham e têm a pele desprendida; beterrabas e batatas doces, para favorecer seu descascamento. Os alimentos são submetidos ao branqueamento para a inativação da catalase e/ou peroxidase.

Antes do advento do branqueamento, o mercado de hortaliças apertizadas era limitado devido à baixa aceitação do produto, em consequência das alterações das características organolépticas do mesmo. Na apertização onde o alimento é tratado em recipientes hermeticamente fechados, para produtos com pH inferior a 4,5 (frutas e tomates), o processo é realizado à temperatura de cocção e, nos alimentos pouco ácidos (leite, feijão e milho), a destruição é dada a elevada temperatura, sob pressão de vapor. O branqueamento varia com a consistência e com o tamanho do material, podendo variar de 2 a 10 minutos, a temperatura entre 70°C e 80°C.

Para avaliar a eficiência do branqueamento, a atividade da enzima termorresistente presente no produto, é utilizada como indicador biológico do processo.

Outro processo utilizado freqüentemente na descontaminação de alimentos é a pasteurização ($\text{pH} < 4,5$, $A_a > 0,98$), processo térmico criado por Pasteur em 1864, que tem por objetivo a eliminação da microbiota patogênica. A temperatura não ultrapassa de 100°C, podendo ser por imersão em água quente, calor seco, vapor, corrente elétrica, radiação ionizante ou “*ultra high pressure*” (UHP).

A pasteurização é indicada para produtos termossensíveis quando as qualidades dos mesmos são afetadas em tratamentos a temperaturas mais altas. A temperatura e o tempo de tratamento estão relacionados com a carga microbiana estimada nos produtos. Os processos de branqueamento, pasteurização e higienização podem ser realizados sob as mesmas condições dependendo do produto e do destino do mesmo.

TIJSKENS (1999) pesquisou a atividade de pectinesterase (PME) em pêssegos, após serem submetidos ao branqueamento. Observou-se que para temperaturas elevadas a taxa de perda de atividade foi maior do que para os tratamentos à temperaturas mais baixas. Este sistema é afetado em temperaturas próximas à 60°C.

Segundo BASAK (1999), a recuperação da PME é geralmente utilizada como indicador da pasteurização adequada do suco de laranja (pH=3,2-3,7). A enzima apresentou maior termoresistência do que células vegetativas de microorganismos que venham sobreviver ao processo e desenvolver durante a conservação. O método convencional de pasteurização de suco de laranja a temperatura de 90°C por 1 minuto é utilizado para a inativação da PME (EARGEMAN AND HOUSE, 1976). Em substituição ao método térmico, a pasteurização por "UHP" tem sido proposta no processamento de produtos termossensíveis com o objetivo de manter as características naturais do suco de laranja (cor e aroma).

A enzima PME também atua em tomates. LARATTA *et al.*, (1995), verificaram que a termoestabilidade da PME aumenta com o aumento do valor de pH.

A ausência de atividade da peroxidase em hortaliças e frutas branqueadas, é uma indicação de que as enzimas foram inativadas durante o processo garantindo a manutenção das características organolépticas e de cor durante o armazenamento do produto.

Visando a conservação de produtos que constituem substratos favoráveis para microorganismos mesófilos (atividade ideal na temperatura de 35°C e não suportam calor superior à temperatura de 65°C) indica-se a pasteurização. Este processo também é indicado para a conservação de

líquidos de pH ácido (vinhos, sucos de frutas, $\text{pH} < 4,5$), pois, são meios impróprios para a proliferação bacteriana, mas sujeitos à proliferação de fungos e leveduras, que são destruídos no processo de pasteurização.

A higienização é indicada para o leite e seus derivados ($\text{pH} > 4,5$; $\text{Aa} > 0,98$). Historicamente, a pasteurização do leite foi baseada na contaminação por *Mycobacterium tuberculosis* e *Coxiella burnetti*, mas com a constante descoberta de novos patógenos, a relação tempo/temperatura sempre deve ser estudada. Dependendo da carga microbiana no leite, se esta for elevada, o tratamento pode ocasionar alguns inconvenientes tais como a alteração de sabor e cor (caramelização da lactose).

Para a higienização do leite, são recomendados dois processos:

- (a) lenta a baixa temperatura (LTLT) – 63°C por 30 segundos;
- (b) rápida a alta temperatura (HTST) – 72°C por 15 segundos.

O principal objetivo da higienização de leite para a fabricação de queijos, é a eliminação de patógenos presentes no leite (GRAPPIN, 1997). Por razões higiênicas, na fabricação de queijos utiliza-se leite submetido à temperatura de 63°C – 65°C por 30–32 segundos ou 72°C – 75°C por 15–30 segundos.

A fosfatase alcalina, uma fosfomonoestearase ligada à gordura do leite, perde a sua atividade a temperaturas acima de 70°C por 15 segundos. Desta forma, é inativada com a pasteurização (GRAPPIN, 1997). O teste negativo da fosfatase alcalina é utilizado como índice do tratamento adequado, assim como a recuperação da atividade da lactoperoxidase é uma indicação de que o leite não foi aquecido à temperatura superior a 80°C (GRIFFITHS, 1986; VILLAMIEL, 1997).

Listeria monocytogenes é um patógeno encontrado no leite não processado que pode ser eliminado após pasteurização à temperatura superior a 69°C por 16 segundos. Utilizando *L. innocua* como padrão, PIYASENA (1998) confirmou o fato com seu experimento onde variou a temperatura entre $60,5^{\circ}\text{C}$ e $69,5^{\circ}\text{C}$ com incrementos de $0,5^{\circ}\text{C}$ por tempos de retenção no tubo do pasteurizador por 3, 10, 16, 30 e 60 segundos. Por simulação gráfica, obteve-se para a retenção de 16 segundos (padrão) a

redução de 4,17 ciclos logarítmicos para a temperatura de 67°C; 11,6 ciclos para a temperatura de 69,5°C e de 28,1 ciclos logarítmicos de redução para a temperatura de 72°C.

Segundo EAGERMAN (1976), que estudou a inativação da pectinestearase em sucos cítricos, obteve as seguintes temperaturas de processo: Hamlin $T = 87,5^{\circ}\text{C}$; Pineapple $T = 87,7^{\circ}\text{C}$; Valencia $T = 90^{\circ}\text{C}$, Duncan grapefruit, $T = 85,5^{\circ}\text{C}$, com os respectivos valores $z (^{\circ}\text{C})$: 4,83, 5,05, 6,77, 5,16.; e valor $D_{90\text{C}} = 1,0$ min para laranja Valencia. A pectinesterase, utilizada como indicador biológico na pasteurização de suco de laranja, apresenta parâmetros cinéticos semelhantes às células vegetativas e microorganismos.

As enzimas PME e fosfatase alcalina são indicadores biológicos da pasteurização do suco de laranja e higienização do leite, respectivamente.

Importância deve ser dada ao correto processamento de alimentos, pois em estudo de hambúrgueres produzidos no Brasil (SILVEIRA, 1999), foi indicada a presença de cepas de *E. coli* O 157:H7, a qual, as verocitotoxinas produzidas causam a colite hemorrágica, similar à disenteria provocada por *Shigella*. As principais fontes desta cepa de *E. coli* são a carne bovina e de ovelhas, seus derivados, leite cru, vegetais, maionese e suco de maçã. Além disso, foram identificadas também cepas de *E. cloacae*, *K. Pneumoniae*, *Citrobacter* sp., entre outras, que, se comparadas à ocorrência de *Salmonella* não são tão expressivas.

Lactoperoxidase também pode ser utilizada como indicador para processos térmicos às temperaturas superiores à 72°C. GRIFFITHS (1986) comparou a lactoperoxidase com outras enzimas do leite, e sugere que esta possa ser utilizada como parâmetro de pasteurização à temperatura de 76°C por 15 segundos. Outros autores verificaram inativação de 99% no intervalo de temperatura 79°C–82°C (McKELLAR, 1996).

Aflatoxinas são compostos altamente tóxicos, mutagênicos, teratogênicos e carcinogênicos, que ocasionam carcinogênese hepática. A exposição a aflatoxinas se dá pela ingestão alimentos contaminados de origem vegetal ou animal. São metabólitos secundários produzidos por

espécies de *Aspergillus*, especificamente *A. flavus* e *A. parasiticus*, que são encontrados no ar, no solo, em tecidos vegetais contaminados, assim também em fígado de animais. Sementes de algodão, amendoim e arroz podem ser contaminados por *Aspergillus sp*: a temperatura de crescimento de *A. parasiticus* é entre 25°-35°C e, para *A. flavus*, a temperatura ideal de crescimento é de 28°-30°C, com umidade de cerca de 10%, com umidade relativa de 82%, sendo de 15 a 35% de umidade no amendoim para a produção de aflatoxina. Aflatoxinas têm alta temperatura de decomposição ficando entre 237°C a 306°C. O cozimento dos alimentos à 150°C não inativa determinados tipos de aflatoxina. A extensão da inativação irá depender da carga contaminante inicial, aquecimento, temperatura e tempo. A degradação também irá depender do pH, da umidade e a força iônica do produto. Estudos anteriores, mostram que quanto maior a umidade do alimento, mais facilmente a toxina pode ser inativada pelo calor. Um exemplo é sementes de algodão com 30% de umidade a 100°C por 1 hora, degrada 74,8% de aflatoxinas, enquanto que uma amostra com 6,6% de umidade, apenas 32,7% de aflatoxinas são degradadas.

RUSTOM (1997) cita os efeitos do pH (5,0, 8,0 e 10,2), temperatura (121°C, 130°C e 140°C) e tempo de aquecimento (5 segundos, 20 segundos e 15 minutos), estudados anteriormente. Tratamento ao valor de pH 8,0 não foi efetivo contra as aflatoxinas. No entanto, tratamentos térmicos ao valor de pH=10,2, 130°C por 20 segundos e ao valor de pH=10,2, 121°C, 15 minutos, reduziu a atividade de aflatoxinas em 78% e 88%, respectivamente. Os tratamentos ao valor de pH 5,0, 130°C, 20 segundos, e, pH 5,0, 121°C, 15 minutos reduziram a atividade em 76% e 73%, respectivamente.

Foram identificadas também cepas de *Listeria* e de *Clostridium* nas amostras processadas após várias semanas de armazenamento.

Tratamento térmico também pode afetar as propriedades dos alimentos. O aquecimento excessivo destes alimentos faz com que se perca propriedades organolépticas. Mudanças de coloração em alimentos podem ser utilizadas como indicador para avaliar a extensão da deterioração devido ao processo térmico (ÁVILA, 1999).

OBJETIVOS

1. Objetivo Geral

O objetivo principal do trabalho foi a determinação dos parâmetros cinéticos de destruição térmica (valor D_{Tr} , valor z , coeficiente Q_{10} e Energia de Ativação, E_a) da proteína verde recombinante, *GFPuv*, em meio de diferentes soluções-tampão. Analisar a influência de cada composição dos tampões na resistência térmica da proteína, para avaliar a possibilidade de utilização posterior como eficiente indicador biológico nos processos de esterilização.

O trabalho objetivou a análise da temorroresistência da proteína *GFPuv* em diferentes valores de pH, na faixa de 5,0 a 9,0. Para a obtenção dos valores de pH, foram utilizados três sistemas de solução-tampão diferentes:

- (i) tampão ácido acético/acetato de sódio 10mM, na faixa de pH entre 5,0 a 7,0;
- (ii) tampão fosfato de sódio monobásico/fosfato de sódio dibásico 10mM na faixa de pH de 6,0 a 8,0;
- (iii) tampão tris- HCl 10mM na faixa de pH entre 7,0 e 9,0.

Para se alcançar o objetivo final, foram realizados estudos adicionais:

1. Transformação de células competentes de *Escherichia coli DH5- α* com o plasmídeo *pGFPuv*, e conseqüente expressão da proteína verde fluorescente recombinante (*GFPuv*), apresentado no anexo 05;
2. Extração física (sonicação/congelamento e descongelamento) e química (método de três fases em partição – *TPP: Three- phase-partitioning extraction*) da proteína *GFPuv* expressa por células de *E. coli*, apresentado no anexo 05;

3. Parâmetros cinéticos do crescimento de células competentes de *Escherichia coli* DH5- α , e da expressão de *GFPuv* durante o desenvolvimento destas células, apresentado no anexo 06;
4. Avaliação da purificação da *GFPuv* pelo método de cromatografia de interação hidrofóbica, apresentado no anexo 07;
5. Parâmetros de estabilidade térmica da proteína *GFPuv* extraída de células competentes de *E. coli*, isolada e purificada em laboratório em relação às características térmicas da proteína padrão *GFPuv*, adquirida da Clontech®. Trabalho apresentado no anexo 08.

MATERIAIS E MÉTODOS

1. Microorganismo Utilizado

Células de *Escherichia coli* DH5- α , transformadas com o plasmídeo *pGFPuv* expressando a proteína verde fluorescente, *GFPuv* (Anexo 05).

Figura 05. Células de *E. coli* DH5- α expressando a *GFPuv*

2. Solução de *GFPuv*

A Proteína Verde Fluorescente (*GFPuv*) extraída de células de *E. coli* DH5- α pelo processo químico de partição em três fases (*Three Phase Partitioning*) e purificada em coluna cromatográfica de interação hidrofóbica (HIC) (Anexo 05 e Anexo 06), foi armazenada em solução-tampão Tris – EDTA 10mM, até o momento de uso.

Figura 06. *GFPuv* em solução-tampão tris-EDTA 10mM

3. Soluções-Tampão (MORITA & ASSUMPÇÃO, 2001)

3.1. Tampão Acetato de Sódio/Ácido Acético 10 mM

Para análise da resistência térmica da *GFPuv* estudou-se o intervalo de pH entre 5,0 e 7,0, em solução-tampão acetato.

3.1.1. Solução de Ácido Acético

Diluiu-se 0,58mL de ácido acético glacial (Synth®) em 900mL de água MILLI-Q, completando o volume para 1000 mL.

Esta solução apresentou pH em torno de 3,4.

3.1.2. Solução de Acetato de Sódio

Dissolveu-se 1,3608g de acetato de sódio trihidratado (Synth®) em 900mL de água MILLI-Q, completando o volume para 1000 mL.

Esta solução apresentou pH em torno de 7,4.

Adicionou-se a solução de ácido acético sobre a solução de acetato de sódio, homogeneizando e medindo o pH, com auxílio de pHmetro.

Retirou-se alíquotas da solução, conforme foram sendo ajustados os pHs necessários.

3.2. Tampão Fosfato de Potássio Monobásico/Fosfato de potássio Dibásico 10 mM

Para análise da resistência térmica da *GFPuv* estudou-se o intervalo de pH entre 6,0 e 8,0, em solução-tampão fosfato.

3.2.1. Solução de Fosfato de Potássio Monobásico 10mM

Dissolveu-se 1,3609g de fosfato de potássio monobásico (Merck®) em 900mL de água MILLI-Q, completando o volume para 1000 mL.

Esta solução apresentou pH em torno de 4,5.

3.2.2. Solução de Fosfato de Potássio Dibásico 10mM

Dissolveu-se 1,7418g de fosfato de potássio dibásico (Merck®) em 900mL de água MILLI-Q, completando o volume para 1000 mL.

Esta solução apresentou pH em torno de 8,7.

Adicionou-se a solução de fosfato de potássio monobásico sobre a solução de fosfato de potássio dibásico, homogeneizando e medindo o pH, com auxílio de pHmetro.

Retirou-se alíquotas da solução, conforme foram sendo ajustados os pHs necessários.

3.3. Tampão de Tris-HCl 10mM

Para análise da resistência térmica da *GFPuv* estudou-se o intervalo de pH entre 7,0 e 9,0, em solução-tampão tris-HCl.

3.3.1. Solução de Tris 10mM

Dissolveu-se 1,211g de tris (Serva®) em 900 de água MILLI-Q, completando o volume para 1000 mL.

Esta solução apresentou pH em torno de 10,14.

3.2. Solução de Ácido Clorídrico 10mM (pH 1,4)

Diluiu-se 0,85mL de ácido clorídrico glacial (Synth®) em 900mL de água MILLI-Q, completando o volume para 1000 mL.

Adicionou-se a solução de ácido clorídrico sobre a solução de tris, homogeneizando e medindo o pH, com auxílio de pHmetro.

Retirou-se alíquotas da solução, conforme foram sendo ajustados os pHs necessários.

4. Metodologia

4.1. Acondicionamento da proteína e Tratamento térmico (Anexo 08)

As soluções-tampão foram preparadas e acondicionadas a temperatura de 4°C até a estabilização dos sistemas (em torno de 24 horas).

As amostras de *GFPuv* foram diluídas em alíquotas de 1,0mL dos diferentes tampões em concentrações que variaram de 5,0µg a 9,5µg de *GFPuv*/mL de solução. Determinou-se a concentração inicial de *GFPuv* em solução. As amostras permaneceram em repouso por 24 horas em geladeira (4°C) para estabilização do sistema e do pH. Após este período, as amostras foram retiradas da geladeira e permaneceram em repouso até que a temperatura das soluções atingisse a temperatura ambiente (cerca de 60 minutos).

A concentração de *GFPuv* nas amostras foi quantificada novamente e o pH da solução-tampão lido novamente, sendo este considerado como o pH de acondicionamento do sistema solução-tampão e proteína.

Determinaram-se os valores de pH a 25°C, das soluções-tampão por pHmetro/conduvímeter digital (Accumet AR20, Fisher Scientific, Pittsburgh, USA) calibrado e aferido, em soluções-tampão padrão (Merck®) em valores de pH iguais a 4,0, 7,0 e 9,0.

Como visto em experimentos anteriores (Anexo 08), a estabilidade da proteína isolada não é alterada expressivamente em temperaturas iguais ou inferiores à 70°C. Diante disto, foram realizados tratamentos térmicos em fluorímetro (Shimadzu RF 5301, Shimadzu Corporation, Kyoto, Japan) nas temperaturas de 75°C, 80°C, 85°C, 90°C e 95°C nos diversos tampões e pHs.

Cada alíquota de *GFPuv* foi transferida para cubeta de quartzo (caminho ótico (3mm x 10mm) e 45mm de altura) e esta recoberta com *parafilm* para evitar possível evaporação. A cubeta foi colocada no respectivo compartimento e o tratamento térmico iniciado nos comprimentos

de onda de 394nm e de 509nm para a excitação e emissão da fluorescência, respectivamente.

O fluorímetro é conectado a um banho de água (Thermo – bath TB 85, (P/N 200- 65022, Shimadzu Corporation, Kyoto, Japan)), o qual permite circulação de água a temperatura constante para o compartimento onde se encontra a amostra de *GFPuv*, tornando o tratamento térmico homogêneo.

Os dados de intensidade de fluorescência foram coletados em intervalos de tempo pré-determinados, dependendo da temperatura de tratamento, sendo a cada 20 segundos para as temperaturas de 75°C e 80°C e a cada 5 segundos para as temperaturas de 85°C, 90°C e 95°C.

Figura 07. Sistema de banho de água acoplado a espectrofluorímetro para realização dos tratamentos térmicos

4.2. Curva padrão da relação entre intensidade de fluorescência da *GFPuv* e a concentração em μg de *GFPuv*/mL

Para a obtenção dos dados, considerou-se a tendência geral das retas de decaimento da intensidade de fluorescência da proteína *GFPuv*.

Pela equação de reta obtida pela representação gráfica da concentração da *GFPuv* padrão (Clontech®) pela intensidade de fluorescência ($\lambda_{\text{exc}} = 394\text{nm}$ e $\lambda_{\text{em}} = 509\text{nm}$), converteu-se os valores de intensidade de fluorescência em concentração de *GFPuv*, expressa em $\mu\text{g}/\text{mL}$ de solução e, a partir destes dados, estimados os parâmetros de resistência térmica: valor D, valor z, valor Q_{10} e Energia de ativação.

Figura 08. Curva padrão da concentração de *GFPuv* ($\mu\text{g}/\text{mL}$) em função da Intensidade de fluorescência ($\lambda_{\text{exc}} = 394\text{nm}$ e $\lambda_{\text{em}} = 509\text{nm}$)

5. Metodologia da análise de dados

Determinação dos parâmetros cinéticos de estabilidade térmica da proteína

5.1. Tempo de Redução decimal (Valor D) e Taxa constante de inativação (valor k)

A inativação da concentração conhecida de proteínas é considerada de primeira ordem. O fenômeno de inativação térmica pode ser representado pelo modelo de curva linearizada, semi-logarítmica de concentração de proteínas não denaturadas. A representação gráfica do logaritmo decimal da concentração residual de proteína em relação ao tempo de exposição à temperatura constante, foi exemplificada abaixo para o tratamento da *GFPuv* em solução-tampão acetato, à temperatura de 75°C, ao valor de pH 6,13, conforme figura 09.

Figura 09. Representação gráfica para o cálculo do tempo de redução decimal (valor D) da *GFPuv* em solução-tampão acetato, pH 6,13.

Para o modelo de curva semi-logarítmica da concentração residual de *GFPuv*, o tempo necessário para o decaimento de 90% da concentração

inicial de proteínas, a uma dada temperatura de referência (T_R) é denominado tempo de redução decimal ou Valor D.

O valor D_{TR} foi calculado pelo inverso negativo do coeficiente angular da reta resultante da regressão linear aplicado à região linear da curva de decaimento, podendo ser representado pela equação:

$$\log_{10} C_f = \log_{10} C_o - (1/D_{TR}) \cdot t \quad \text{ou}$$
$$= \log_{10} C_o - (k/2.303) \cdot t, \quad \text{portanto:}$$

$$\text{Valor D} = 2.303/k.$$

onde: C_o = concentração inicial de *GFPuv* ($\mu\text{g/mL}$);

C_f = concentração final de *GFPuv* ($\mu\text{g/mL}$);

k = taxa constante de inativação (h^{-1});

t = tempo de exposição à temperatura (s).

Para o exemplo citado, tem-se que:

$$\log_{10} C_f = \log_{10} C_o - (1/D_{TR}) \cdot t$$

$$\log_{10} \text{GFPuv} = 0,823 - 0,0011 \cdot t, \quad \text{ou seja,}$$

$$0,0011 = 1/D_{TR}, \quad \text{portanto,}$$

$$D_{TR} = 1/0,0011 = 909,0 \text{ s} = 15,1 \text{ min}$$

5.2. Valor z e Coeficiente Q_{10}

O valor z, expresso em graus Celsius ($^{\circ}\text{C}$), expressa o intervalo de temperatura necessário para a redução de um ciclo logarítmico no Valor D. É determinado pela equação de relação entre o logaritmo do Valor D e as correspondentes temperaturas de tratamento, como exemplificado na figura 10, para o sistema de tampão acetato em pH 6,0, ou pela equação:

$$\text{Log}_{10} D_2 = \text{Log}_{10} D_1 - 1/z * (T_2 - T_1)$$

onde: D_1 = valor D à temperatura de tratamento, T_1 ;

D_2 = valor D à temperatura de tratamento, T_2

Figura 10. Representação gráfica para o cálculo do valor z para o sistema de *GFPuv* em tampão acetato, pH 6,0.

Para o exemplo citado, tem-se que:

$$\text{Log}_{10} D = 3.5855 - 0.0323 * (T_2 - T_1), \text{ ou seja,}$$

$$0,0323 = 1/z, \text{ portanto,}$$

$$\text{valor } z = 1/0,0323 = 30,96^{\circ}\text{C}$$

O coeficiente Q_{10} , fator que avalia a dependência da taxa de inativação constante, "k", com o aumento de 10°C na temperatura de tratamento (T , °C), pode ser estimado pela relação:

$$Q_{10} = [k_{(T+10)} / k_T],$$

ou relacionado ao valor z:

$$Q_{10} = (10^{10/z})$$

5.3. Energia de Ativação (Ea)

A Energia de Ativação foi estimada utilizando-se a equação de Arrhenius, a qual foi aplicada para avaliar a dependência entre a taxa de inativação constante (k) com a temperatura, podendo ser representada graficamente (Figura 11) ou pela equação:

$$\log k_1 = \log k_2 - \{(E_a / 2.303 \cdot R) \cdot [(1/T_1) - (1/T_2)]\}, \text{ logo:}$$

$$\alpha = E_a / (2.303 \cdot R) \text{ ou } E_a = \alpha \cdot 2.303 \cdot R$$

onde: k = taxa constante de inativação (h^{-1});
 T_1 e T_2 = temperaturas de tratamento em graus Kelvin (K)
 R = constante universal dos gases (1,987 calorias / mol*K),

Figura 11. Representação gráfica para o cálculo da Energia de Ativação para o sistema de tampão acetato, pH 6,0

Para o exemplo citado, tem-se que:

$$\begin{aligned} \log k &= 11,171 - 4211,2 \cdot (1/T_1 - 1/T_2), \text{ ou seja,} \\ 4211,2 &= E_a / (2,303 \cdot 1,987), \text{ portanto,} \\ E_a &= 4211,2 \cdot 2,303 \cdot 1,987 = 19,27 \text{ kcal/mol} \end{aligned}$$

RESULTADOS E DISCUSSÃO

1. Tratamento térmico

1.1. Determinação do Tempo de Redução Decimal (Valor D)

A variação da estabilidade térmica da *GFPuv* em soluções-tampão e valores de pH ajustados e estabilizados foi estudada para as temperaturas de 75°C, 80°C, 85°C, 90°C e 95°C.

Para cada curva de decaimento da concentração de *GFPuv* foi possível a determinação do tempo de redução decimal, ou valor D, através do inverso negativo do coeficiente angular da reta ajustada à região linear.

Os valores D determinados para a proteína *GFPuv* mostraram-se proporcionais a determinada faixa de pH, independente das composições das soluções-tampão. Observou-se que a estabilidade térmica da *GFPuv* aumentou com o incremento gradual nos valores de pH das soluções, e foi reduzida com o aumento da temperatura. A estabilidade da proteína foi avaliada em termos de valor D.

As curvas de decaimento da concentração de *GFPuv* para as temperaturas e soluções-tampão estudadas, em relação aos valores de pH selecionados, são apresentadas para:

- (a) tampão acetato/ácido acético 10mM, ao valor de pH 6,0 (figura 12);
- (b) tampão fosfato de potássio monobásico/dibásico 10mM, ao valor de pH 6,5 (figura 13);
- (c) tampão tris-HCl 10mM, para os valores de pH 7,5, 8,0 e 8,5 (figuras 14, 15, 16).

Figura 12. Curvas de decaimento da concentração de GFPuv ($\mu\text{g GFPuv/mL}$) em solução-tampão acetato 10mM, pH 6,0

Figura 13. Curvas de decaimento da concentração de GFPuv ($\mu\text{g GFPuv/mL}$) em solução-tampão fosfato 10mM, pH 6,5

Figura 14. Curvas de decaimento da concentração de *GFPuv* ($\mu\text{g GFPuv/mL}$) em solução-tampão tris-HCl 10mM, pH 7,5

Figura 15. Curvas de decaimento da concentração de *GFPuv* ($\mu\text{g GFPuv/mL}$) em solução-tampão tris-HCl 10mM, pH 8,0

Figura 16. Curvas de decaimento da concentração de *GFPuv* ($\mu\text{g GFPuv/mL}$) em solução-tampão tris -HCl 10mM, pH 8,5

Para os tratamentos térmicos realizados às temperaturas de 75°C, 80°C e 85°C, o perfil das curvas de decaimento da concentração de *GFPuv* mostrou proporcionalidade linear em relação ao período de exposição.

Para os tratamentos térmicos realizados às temperaturas de 90°C e 95°C, a curva de decaimento mostrou perfil tendendo a formar dois comportamentos distintos os quais não foram considerados para o cálculo do valor D. Utilizou-se a média dos valores D calculados para cada segmento de reta linearizada. Os valores D médios (tendência geral) e referentes aos segmentos de reta, tendência 1 e 2, estão apresentados no Anexo 01.

1.1.1. Tratamento térmico à temperatura de 75°C

A Tabela 3 e respectivos histogramas (figuras 17, 18 e 19) mostram para a temperatura de 75°C, que a estabilidade da *GFPuv* em solução tampão acetato foi maior para valores de pH > 5,66 ($D_{pH\ 5,66} = 7,93$ min), atingindo máxima estabilidade ao valor de pH 7,0 ($D_{pH\ 6,89\ e\ 7,01} = 41,6$ min).

Em tampão fosfato, a estabilidade da *GFPuv* foi duas vezes maior na faixa de pH entre 6,81 e 7,74 ($D_{pH\ 6,81 - 7,74} = 83,3$ min), do que o valor D apresentado para a *GFPuv* em tampão acetato ($D_{pH\ 6,89 - 7,01} = 41,6$ min), atingindo valor máximo ao valor de pH 7,84 ($D_{pH\ 7,84} = 166,6$ min).

A *GFPuv* apresentou estabilidade em tampão Tris-HCl entre os valores de pH 7,67 a 8,50 ($D_{pH\ 7,67} = 83,33$ min e $D_{pH\ 8,50} = 166,6$ min (valor máximo)), sendo similar à estabilidade em tampão fosfato na mesma faixa de pH. Para valores de pH > 8,50, não houve reprodutibilidade do valor D para *GFPuv* ($D_{pH\ 8,65} = 20,83$ min e $D_{pH\ 8,90} = 83,3$ min).

Tabela 03. Valores D obtidos no tratamento térmico da *GFPuv*, exposta à temperatura de 75°C

Tampão	pH	Equação $\text{Log}_{10} \text{GFPuv}$	R^2	Valor D (min)
Acetato 75°C	4,98	$= -0,0016*t + 0,1446$	0,97	10,41
	5,18	$= -0,0027*t + 0,4834$	0,98	6,17
	5,32	$= -0,0019*t + 0,4355$	0,996	8,77
	5,50	$= -0,0033*t + 0,6889$	0,993	5,05
	5,66	$= -0,0021*t + 0,7531$	0,98	7,93
	6,13	$= -0,0011*t + 0,823$	0,999	15,10
	6,49	$= -0,0006*t + 0,7745$	0,995	27,70
	6,89	$= -0,0004*t + 0,7335$	0,992	41,60
	7,01	$= -0,0004*t + 0,7237$	0,94	41,60
Fosfato 75°C	5,67	$= -0,0014*t + 0,2214$	0,998	11,90
	5,90	$= -0,0012*t + 0,6284$	0,98	13,88
	6,19	$= -0,002 *t + 0,7173$	0,994	8,33
	6,19	$= -0,0013*t + 0,834$	0,993	12,82
	6,28	$= -0,002 *t + 0,7483$	0,996	8,33
	6,40	$= -0,001 *t + 0,7535$	0,991	16,66
	6,50	$= -0,0005*t + 0,6165$	0,95	33,33
	6,81	$= -0,0002*t + 0,617$	0,93	83,33
	7,11	$= -0,0003 *t + 0,6441$	0,92	55,55
	7,20	$= -0,0002*t + 0,6096$	0,93	83,33
	7,34	$= -0,0002 *t + 0,6511$	0,94	83,33
	7,36	$= -0,0002 *t + 0,6609$	0,95	83,33
	7,50	$= -0,0002*t + 0,5812$	0,86	83,33
	7,60	$= -0,0002*t + 0,5894$	0,94	83,33
	7,74	$= -0,0002*t + 0,5584$	0,85	83,33
	7,84	$= -0,0001*t + 0,5652$	0,92	166,66

Tabela 03. Valores D obtidos no tratamento térmico da *GFPuv*, exposta à temperatura de 75°C (continuação)

Tampão	pH	Equação $\text{Log}_{10} \text{GFPuv}$	R^2	Valor D (min)
Tris – HCl 75°C	6,67	$= -0,0004 *t + 0,6779$	0,91	41,60
	6,91	$= -0,0017 *t + 0,7462$	0,997	9,80
	7,14	$= -0,0005 *t + 0,6590$	0,96	33,33
	7,41	$= -0,0006 *t + 0,6909$	0,94	27,70
	7,61	$= -0,0003 *t + 0,6036$	0,90	55,55
	7,67	$= -0,0002 *t + 0,533$	0,87	83,33
	8,05	$= -0,0002 *t + 0,6285$	0,93	83,33
	8,5	$= -0,0001 *t + 0,5785$	0,92	166,66
	8,65	$= -0,0008 *t + 0,7003$	0,96	20,83
	8,90	$= -0,0002 *t + 0,5987$	0,89	83,33

Figura 17. Valores D obtidos para a proteína *GFPuv* exposta à temperatura de 75°C, em solução-tampão acetato 10mM

Figura 18. Valores D obtidos para a proteína *GFPuv* exposta à temperatura de 75°C, em solução-tampão fosfato 10mM

Figura 19. Valores D obtidos para a proteína *GFPuv* exposta à temperatura de 75°C, em solução-tampão tris-HCl 10mM

1.1.2. Tratamento térmico à temperatura de 80°C

Analizando-se a Tabela 04 e respectivos histogramas (figuras 20, 21 e 22), a estabilidade em solução tampão acetato foi maior aos valores de pH acima de 6,0 ($D_{pH\ 6,15} = 10,14$ min), atingindo valor máximo em pH 7,0 ($D_{pH\ 7,03} = 20,80$ min)

Em tampão fosfato, a *GFPuv* apresentou estabilidade térmica aos valores de pH > 5,97 ($D_{pH5,97} = 6,94$ min), atingindo máxima estabilidade em pH 8,0 ($D_{pH7,91} = 83,3$ min). Em pH 7,0, o valor D obtido ($D_{pH\ 7,13} = 55,55$ min) foi cerca de 2,5 vezes maior em relação ao valor D máximo ($D_{pH\ 7,03} = 20,80$ min) obtido para o tampão acetato.

Em tampão tris-HCl, a *GFPuv* apresentou maior estabilidade em pH 8,45 ($D_{pH8,45} = 55,55$ min), valor semelhante aquele definido em tampão fosfato, pH 7,0 ($D_{pH7,13} = 55,55$ min). Ao valor de pH 7,5 ($D_{pH7,55} = 20,83$ min), a *GFPuv* em tampão tris-HCl apresentou o mesmo valor D determinado em tampão acetato, pH 7,0 ($D_{pH7,03} = 20,80$ min).

Tabela 04. Valores D obtidos no tratamento térmico da *GFPuv* exposta à temperatura de 80°C.

Tampão	pH	Equação $\text{Log}_{10} \text{GFPuv}$	R^2	Valor D (min)
Acetato 80°C	5,18	$= -0,0029*t + 0,5246$	0,94	5,74
	5,64	$= -0,0023*t + 0,7025$	0,95	7,24
	6,15	$= -0,0016*t + 0,8252$	0,990	10,14
	6,53	$= -0,0011*t + 0,8099$	0,993	15,10
	6,92	$= -0,0008*t + 0,7793$	0,997	20,80
	7,03	$= -0,0008*t + 0,7742$	0,996	20,80
Fosfato 80°C	5,97	$= -0,0024*t + 0,8265$	0,96	6,94
	6,20	$= -0,0018*t + 0,8005$	0,97	9,26
	6,22	$= -0,0016*t + 0,7889$	0,993	10,41
	6,68	$= -0,0007*t + 0,7797$	0,993	23,8
	7,13	$= -0,0003*t + 0,6592$	0,991	55,5
	7,55	$= -0,0002*t + 0,5491$	0,97	83,3
Tris – HCl 80°C	7,91	$= -0,0002*t + 0,6307$	0,993	83,3
	7,50	$= -0,0008*t + 0,8060$	0,97	20,83
	8,08	$= -0,0004*t + 0,7185$	0,994	41,6
	8,45	$= -0,0003*t + 0,7647$	0,98	55,55
	8,84	$= -0,0003*t + 0,6231$	0,992	55,55

Figura 20. Valores D obtidos no tratamento térmico da *GFPuv* à temperatura de 80°C, em solução-tampão acetato 10mM

Figura 21. Valores D obtidos no tratamento térmico da *GFPuv* à temperatura de 80°C, em solução-tampão fosfato 10mM

Figura 22. Valores D obtidos no tratamento térmico da *GFPuv* à temperatura de 80°C, em solução-tampão tris-HCl 10mM.

1.1.3. Tratamento térmico à temperatura de 85°C

Em tampão acetato, a estabilidade térmica da *GFPuv* aumentou gradualmente aos valores de pH > 6,0 ($D_{pH6,15} = 5,55$ min), atingindo valor máximo em pH 7,0 ($D_{pH7,03} = 8,33$ min) (figura 23).

Em solução-tampão fosfato, a *GFPuv* apresentou máxima estabilidade ao valor de pH 8,0 ($D_{pH7,80} = 18,51$ min) (figura 24). Para o valor de pH 7,0, ($D_{pH7,27} = 13,88$ min) a *GFPuv* apresentou valor D cerca de duas vezes maior do que o valor D obtido em pH 7,0 na solução de tampão acetato ($D_{pH7,03} = 8,33$ min).

Em tampão tris-HCl (figura 25), a *GFPuv* apresentou valor D máximo ao valor de pH $\geq 8,65$ ($D_{pH8,65}$ e $D_{pH8,90} = 27,70$ min). Ao valor de pH 8,50 ($D_{pH8,50} = 18,51$ min), a termoestabilidade da *GFPuv* foi semelhante àquela observada ao valor de pH 8,0, em tampão fosfato ($D_{pH7,80} = 18,51$ min).

Tabela 05. Valores D obtidos para a proteína *GFPuv* em solução-tampão, exposta à temperatura de 85°C

Tampão	pH	Equação $\text{Log}_{10} \text{GFPuv}$	R^2	Valor D (min)
Acetato	4,91	$= -0,0036*t + 0,3091$	0,96	4,62
	5,18	$= -0,0044*t + 0,5979$	0,94	3,78
	5,32	$= -0,0063*t + 0,8464$	0,94	2,64
	5,64	$= -0,0035*t + 0,7394$	0,92	4,76
	6,15	$= -0,003*t + 0,9321$	0,93	5,55
	6,53	$= -0,0024*t + 0,956$	0,96	6,94
	6,92	$= -0,0023*t + 0,9756$	0,97	7,24
	7,03	$= -0,002*t + 0,9131$	0,990	8,33
Fosfato	5,63	$= -0,0041*t + 0,7809$	0,96	4,06
	5,92	$= -0,0036*t + 0,8097$	0,96	4,62
	6,20	$= -0,003*t + 0,936$	0,94	5,55
	6,50	$= -0,0023*t + 0,8172$	0,98	7,24
	6,83	$= -0,0012*t + 0,6488$	0,98	13,88
	7,27	$= -0,0012*t + 0,7509$	0,98	13,88
	7,64	$= -0,0011*t + 0,7146$	0,98	15,15
	7,80	$= -0,0009*t + 0,702$	0,96	18,51
Tris – HCl	7,84	$= -0,0009*t + 0,7757$	0,97	18,51
	7,41	$= -0,0023*t + 0,8146$	0,97	7,24
	7,61	$= -0,0018*t + 0,7802$	0,98	9,25
	8,05	$= -0,0014*t + 0,7687$	0,991	11,90
	8,50	$= -0,0009*t + 0,7168$	0,98	18,51
	8,65	$= -0,0006*t + 0,6202$	0,98	27,7
	8,90	$= -0,0006*t + 0,6202$	0,95	27,7

Figura 23. Valores D obtidos para a proteína *GFPuv* exposta à temperatura de 85°C, em solução-tampão acetato 10mM

Figura 24. Valores D obtidos para a *GFPuv* exposta à temperatura de 85°C, em solução-tampão fosfato 10mM

Figura 25. Valores D obtidos para a proteína *GFPuv* exposta à temperatura de 85°C, em solução-tampão tris-HCl 10mM

1.1.4. Tratamento térmico à temperatura de 90°C

Analisando-se os valores D a 90°C, apresentados na tabela 06 e respectivos histogramas (figuras 26, 27 e 28), observou-se para a *GFPuv* em tampão acetato, aumento gradual da estabilidade da proteína aos valores de pH > 6,50 ($D_{pH\ 6,74 - 7,01} = 5,05$ min). Enquanto que, em tampão fosfato, o valor D apresentou incremento gradual para valores de pH $\geq 6,4$ ($D_{pH6,42} = 4,27$ min), atingindo o valor D máximo ao valor de pH 7,8 ($D_{pH7,80} = 6,94$ min).

O sistema de *GFPuv* em tampão tris-HCl apresentou maior variação nos valores D, atingindo o valor D máximo em pH $\geq 8,4$ ($D_{pH\ 8,38}$ e $D_{pH\ 8,92} = 7,93$ min).

Tabela 06. Valores D obtidos para a proteína *GFPuv* em solução-tampão, exposta à temperatura de 90°C

Tampão	pH	Equação $\text{Log}_{10} \text{GFPuv}$	R^2	Valor D (min)
Acetato 90°C	5,19	$= -0,0051*t + 0,615$	0,93	3,26
	5,72	$= -0,0042*t + 0,8292$	0,91	3,96
	6,25	$= -0,0036*t + 0,9857$	0,91	4,62
	6,74	$= -0,0033*t + 1,022$	0,92	5,05
	6,94	$= -0,0032*t + 1,0107$	0,93	5,20
	7,01	$= -0,0033*t + 1,0464$	0,92	5,05
Fosfato 90°C	6,03	$= -0,0037*t + 0,9005$	0,90	4,50
	6,19	$= -0,0038*t + 0,8923$	0,91	4,38
	6,42	$= -0,0039*t + 0,9621$	0,91	4,27
	6,96	$= -0,0029*t + 1,0053$	0,94	5,74
	7,43	$= -0,0025*t + 0,93$	0,95	6,66
	7,80	$= -0,0024*t + 0,8241$	0,96	6,94
Tris – HCl 90°C	7,27	$= -0,0055*t + 0,8772$	0,95	3,03
	7,42	$= -0,0036*t + 1,0318$	0,90	4,62
	7,50	$= -0,0047*t + 0,9449$	0,93	3,54
	7,72	$= -0,0033*t + 1,0475$	0,92	5,05
	7,74	$= -0,0035*t + 0,9372$	0,94	4,76
	8,05	$= -0,0025*t + 0,9648$	0,95	6,66
	8,05	$= -0,0033*t + 0,9314$	0,96	5,05
	8,37	$= -0,0031*t + 0,9274$	0,97	5,37
	8,38	$= -0,0021*t + 0,8739$	0,96	7,93
	8,60	$= -0,0022*t + 0,9303$	0,96	7,57
	8,64	$= -0,0042*t + 0,8535$	0,98	3,96
	8,82	$= -0,0066*t + 0,993$	0,98	2,52
	8,92	$= -0,0021*t + 0,9195$	0,96	7,93
	9,03	$= -0,003*t + 0,914$	0,97	5,55

Figura 26. Valores D obtidos para a proteína *GFPuv* exposta à temperatura de 90°C, em solução-tampão acetato 10mM

Figura 27. Valores D obtidos para a proteína *GFPuv* exposta à temperatura de 90°C, em solução-tampão fosfato 10mM

Figura 28. Valores D obtidos para a proteína *GFPuv* exposta à temperatura de 90°C, em solução-tampão tris-HCl 10mM

1.1.5. Tratamento térmico à temperatura de 95°C

Analizando a tabela 07 e respectivas figuras 29, 30 e 31, verificou-se variação de 80% nos valores D para a *GFPuv* em solução tris-HCl em relação aos valores D determinados para a *GFPuv* em solução fosfato (41% de variação) e em tampão acetato (29% de variação), à temperatura de 95°C.

Em tampão acetato, os valores D determinados foram semelhantes para os valores de pH $\geq 5,72$ ($D_{pH5,72} = 4,06$ min e $D_{pH7,01} = 4,90$ min).

Em tampão fosfato e valores de pH $>6,2$, os valores D para a *GFPuv* assemelharam-se, variando entre D= 6,17 min (pH= 6,52) e D= 5,37 min, atingindo valor máximo de valor $D_{pH7,82} = 6,41$ min.

Os valores D determinados para a proteína *GFPuv* em tampão tris-HCl demonstraram a instabilidade do sistema na faixa de pH estudada (7,0 - 9,0), sendo os valores $D_{pH7,08} = 1,89$ min e $D_{pH8,97} = 6,94$ min, atingindo valor D máximo em pH 8,92, onde D = 9,80 min.

Tabela 07. Valores D obtidos no tratamento térmico da *GFPuv* exposta à temperatura de 95°C

Tampão	pH	Equação $\text{Log}_{10} \text{GFPuv}$	R^2	Valor D (min)
Acetato 95°C	5,11	$= -0,0036*t + 0,3817$	0,95	4,62
	5,19	$= -0,0048*t + 0,5941$	0,91	3,47
	5,46	$= -0,0049*t + 0,7947$	0,90	3,40
	5,72	$= -0,0041*t + 0,7526$	0,89	4,06
	6,25	$= -0,0038*t + 0,8932$	0,89	4,38
	6,74	$= -0,0037*t + 0,9682$	0,91	4,50
	6,94	$= -0,0034*t + 0,9342$	0,90	4,90
	7,01	$= -0,0034*t + 0,9406$	0,91	4,90
Fosfato 95°C	5,89	$= -0,0044*t + 0,8754$	0,88	3,78
	6,14	$= -0,0038*t + 0,8707$	0,89	4,38
	6,19	$= -0,0037*t + 0,8267$	0,89	4,50
	6,52	$= -0,0027*t + 0,9026$	0,91	6,17
	7,05	$= -0,0029*t + 0,9198$	0,94	5,74
	7,44	$= -0,0028*t + 0,9418$	0,94	5,95
	7,82	$= -0,0026*t + 0,9446$	0,93	6,41
	7,95	$= -0,0029*t + 1,0078$	0,95	5,74
	8,0	$= -0,0031*t + 1,0161$	0,95	5,37
	8,0	$= -0,0031*t + 0,9729$	0,93	5,37

Tabela 07. Valores D obtidos no tratamento térmico da *GFPuv* exposta à temperatura de 95°C (continuação)

Tampão	pH	Equação $\text{Log}_{10} \text{GFP}_{uv}$	R^2	Valor D (min)
Tris – HCl 95°C	7,08	$= -0,0088*t + 1,005$	0,88	1,89
	7,11	$= -0,0079*t + 0,9892$	0,89	2,10
	7,34	$= -0,007*t + 0,9838$	0,89	2,38
	7,44	$= -0,0043*t + 0,9625$	0,88	3,47
	7,68	$= -0,0065*t + 0,9862$	0,90	2,56
	7,72	$= -0,0036*t + 0,9711$	0,89	4,62
	7,89	$= -0,0058*t + 0,9802$	0,91	2,87
	8,05	$= -0,0027*t + 0,9494$	0,92	6,17
	8,34	$= -0,0054*t + 0,958$	0,92	3,08
	8,38	$= -0,002*t + 0,687$	0,96	8,33
	8,55	$= -0,003*t + 0,6113$	0,97	5,55
	8,63	$= -0,0051*t + 0,9639$	0,93	3,26
	8,70	$= -0,0041*t + 0,868$	0,94	4,06
	8,92	$= -0,0017*t + 0,6729$	0,97	9,80
	8,97	$= -0,0024*t + 0,6008$	0,97	6,94

Figura 29. Valores D obtidos no tratamento térmico da *GFPuv* à temperatura de 95°C, em solução-tampão acetato 10mM

Figura 30. Valores D obtidos no tratamento térmico da *GFPuv* à temperatura de 95°C, em solução-tampão fosfato 10mM

Figura 31. Valores D obtidos no tratamento térmico da *GFPuv* à temperatura de 95°C, em solução-tampão tris-HCl 10mM

para os tratamentos térmicos (Anexo 02 e Tabela 07) à temperatura de 75°C, onde a *GFPuv* apresentou melhor termoestabilidade nas três soluções-tampão estudadas.

Figura 33. Valores D (min) da *GFPuv* em solução-tampão acetato 10mM para diferentes valores de pH, à temperatura de 75°C

Para o sistema de tampão acetato a estabilidade da *GFPuv* expressa em termos de valores D (a 75°C), mostrou correlação linear para os valores de pH entre 5,50 e 7,0.

Figura 34. Valores D (min) da *GFPuv* em solução-tampão fosfato 10mM para diferentes valores de pH, à temperatura de 75°C

Em tampão fosfato, a linearidade da relação entre os valores D (min) e o aumento do valor de pH foi entre pH 6,19 e pH 7,20. Para valores de pH acima de 7,20, a estabilidade da proteína independeu do valor de pH da solução-tampão fosfato.

Figura 35. Valores D (min) da *GFPuv* em solução-tampão tris-HCl 10mM para diferentes valores de pH, à temperatura de 75°C

Em tampão tris-HCl 10 mM, observou-se linearidade dos valores D e valores de pH para o intervalo de pH entre 6,91 e 8,50. Para valores de pH > 8,50, verificou-se insconstância de relação entre os valores D obtidos experimentalmente e o aumento gradual do valor de pH da solução-tampão tris-HCl.

Para cada temperatura, as relações entre valores D e valores de pH das soluções-tampão foram analisadas, como exemplificadas para a temperatura de 75°C, sendo as representações gráficas apresentadas no Anexo 02, e as respectivas equações mostradas na tabela 08.

Tabela 08. Equações correlacionando Valores D da *GFPuv* em diferentes valores de pH das soluções-tampão, nas respectivas temperaturas de tratamento

Tampão	Temp. (°C)	Faixa de pH Selecionada	Equação Valor D	R ²
Acetato	75	5,5 - 7,01	= - 138,09 + 25,677*pH	0,97
	80	5,18 - 7,01	= - 41,644 + 8,8106*pH	0,94
	85	4,91 - 7,03	= - 12,1 + 2,8747*pH	0,94
	90	5,19 - 7,01	= - 1,9854 + 1,0318*pH	0,97
	95	5,46 - 7,01	= - 0,9143 + 0,8296*pH	0,91
Fosfato	75	6,19 - 7,20	= - 392,14 + 64,603*pH	0,92
	80	5,97 - 7,91	= - 271,76 + 45,632*pH	0,96
	85	5,63 - 7,84	= - 35,676 + 6,8552*pH	0,94
	90	6,03 - 7,80	= - 5,6922 + 1,6322*pH	0,93
	95	5,89 - 7,82	= - 3,6824 + 1,3041*pH	0,97
Tris-HCl	75	6,91 - 8,50	= - 636,99 + 92,299*pH	0,89
	80	7,50 - 8,84	= - 182,68 + 27,51*pH	0,91
	85	7,41 - 8,90	= - 91,089 + 13,097*pH	0,94
	90	7,27 - 9,03	= - 14,998 + 2,5629*pH	0,77
	95	7,08 - 8,92	= - 30,48 + 4,5551*pH	0,98

A partir das equações de reta, obtidas das correlações lineares entre valores D (min) e valores de pH da solução-tampão, foram estimados valores D para valores de pH: (i) entre 5,0 e 7,5 para tampão acetato; (ii) entre 6,0 e 8,0 para tampão fosfato e (iii) entre 7,0 e 9,0 para tampão tris-HCl, e apresentados na Tabela 09.

Tabela 09. Valores D calculados a partir das equações de reta correspondentes a cada solução-tampão e temperatura de tratamento térmico

Tampão	temp. (°C)	pH					
		6,0	6,5	7,0	7,5	8,0	8,5
Acetato 10mM	75	15,97	28,81	41,65			
	80	11,22	15,62	20,03			
	85	5,15	6,59	8,02			
	90	4,21	4,72	5,24			
	95	4,06	4,48	4,89			
Fosfato 10mM	75		27,78	60,08	92,38	124,68	
	80		24,85	47,66	70,48	93,3	
	85		8,88	12,31	15,74	19,17	
	90		4,92	5,73	6,55	7,37	
	95		4,79	5,45	6,10	6,75	
Tris-HCl 10mM	75			14,97	53,72		
	80			9,89	23,65	37,4	51,16
	85			5,10	7,14	13,69	20,24
	90			2,94	4,22	5,51	6,79
	95			1,72	3,03	4,33	5,64

3. Cálculo dos Valores z para a proteína em soluções-tampão, valores de pH e intervalos de temperatura considerados

O logarítmo decimal dos valores D para a *GFPuv*, estimados para valores de pH específicos das soluções-tampão estudadas, foram correlacionados à temperatura de tratamento (T (°C)). As representações gráficas são apresentadas em Anexo 03, e as equações das funções lineares entre as variáveis, foram dispostas na tabela 10. Os respectivos valores z foram determinados a partir do coeficiente angular das equações de reta ($\text{Log}_{10} D$ versus temperatura °C); e, os valores Q_{10} foram calculados a partir dos valores z ($Q_{10} = 10^{10/z}$), parâmetros estes apresentados na Tabela 10.

Foram selecionadas representações gráficas relacionando $\text{Log}_{10} D$ e temperatura (°C), para cada solução-tampão estudada (figuras 36, 37, 38, 39 e 40).

Figura 36. Correlação entre Log_{10} Valores D (min) e temperaturas de tratamento para *GFPuv* em solução-tampão acetato 10mM, pH 6,0

Figura 37. Correlação entre Log_{10} Valor D (min) e temperatura de tratamento térmico para solução-tampão fosfato 10mM, pH 6,50

Figura 38. Correlação entre Log Valor D (min) e a temperatura de tratamento térmico para solução-tampão tris-HCl 10mM, pH 7,5

Figura 39. Correlação entre Log₁₀ Valor D (min) e a temperatura de tratamento térmico para solução-tampão tris-HCl 10mM, pH 8,0

Figura 40. Correlação entre Log₁₀ Valor D (min) e a temperatura de tratamento térmico para solução-tampão tris-HCl 10mM, pH 8,5

Tabela 10. Valores z calculados a partir das equações de reta correlacionando $\text{Log}_{10} D$ e temperaturas de tratamento, para as soluções-tampão estudadas e, valores Q_{10} estimados a partir dos valores z

Tampão	pH	Equação $\text{Log}_{10} D$	R^2	Valor Z (°C)	Q_{10}
Acetato	6,0	$= -0,0323*t + 3,5855$	0,89	30,96	2,10
	6,5	$= -0,0427*t + 4,5911$	0,91	23,42	2,67
	7,0	$= -0,0489*t + 5,1999$	0,92	20,45	3,08
Fosfato	6,5	$= -0,0446*t + 4,8232$	0,9	22,42	2,79
	7,0	$= -0,0601*t + 6,3163$	0,91	16,64	3,99
	7,5	$= -0,0714*t + 7,4074$	0,87	14,01	5,17
	8,0	$= -0,0727*t + 7,5892$	0,92	13,75	5,34
Tris-HCl	7,0	$= -0,0481*t + 4,8054$	0,996	20,79	3,03
	7,5	$= -0,0649*t + 6,5309$	0,95	15,41	4,46
	8,0	$= -0,0641*t + 6,6294$	0,94	15,60	4,38
	8,5	$= -0,0669*t + 7,0073$	0,93	14,95	4,67

4. Cálculo da Energia de Ativação (E_a)

As taxas de inativação térmica, k , expressando a velocidade de inativação da *GFPuv*, foram calculadas a partir da relação ($k=2,303/D$), para cada solução-tampão estudada e apresentadas nas tabelas 11, 12 e 13. Através da representação gráfica do logarítmo decimal do valor k e o inverso da temperatura absoluta (K), as energias de ativação (E_a , kcal/mol), apresentadas na tabela 14, foram calculadas a partir do coeficiente angular das equações de reta (Tabela 14).

Tabela 11. Coeficientes de inativação térmica (valores k), para *GFPuv* em tampão acetato 10mM

Tampão	Temp. (°C)	Temp (K)	1/K	Valor D (min)	Valor k (min ⁻¹)	Log k
Acetato pH 6,0	75	348	0,00287	15,97	0,144	-0,841
	80	353	0,00283	11,22	0,205	-0,841
	85	358	0,00279	5,15	0,447	-0,688
	90	363	0,00275	4,21	0,547	-0,350
	95	368	0,00272	4,06	0,567	-0,262
Acetato pH 6,5	75	348	0,00287	28,81	0,080	-1,097
	80	353	0,00283	15,62	0,147	-0,831
	85	358	0,00279	6,59	0,349	-0,457
	90	363	0,00275	4,72	0,488	-0,312
	95	368	0,00272	4,48	0,514	-0,289
Acetato pH 7,0	75	348	0,00287	41,65	0,055	-1,257
	80	353	0,00283	20,03	0,115	-0,939
	85	358	0,00279	8,02	0,287	-0,542
	90	363	0,00275	5,24	0,440	-0,357
	95	368	0,00272	4,89	0,471	-0,327

Tabela 12. Coeficientes de inativação térmica (valores k), para *GFPuv* em tampão fosfato 10mM

Tampão	Temp. (°C)	Temp (K)	1/K	Valor D (min)	Valor k (min ⁻¹)	Log k
Fosfato pH 6,50	75	348	0,00287	27,78	0,083	-1,081
	80	353	0,00283	24,85	0,093	-1,033
	85	358	0,00279	8,88	0,259	-0,586
	90	363	0,00275	4,92	0,468	-0,330
	95	368	0,00272	4,79	0,481	-0,318
Fosfato pH 7,0	75	348	0,00287	60,08	0,038	-1,416
	80	353	0,00283	47,66	0,048	-1,316
	85	358	0,00279	12,31	0,187	-0,728
	90	363	0,00275	5,73	0,402	-0,396
	95	368	0,00272	5,45	0,423	-0,374
Fosfato pH 7,5	75	348	0,00287	92,38	0,025	-1,603
	80	353	0,00283	70,48	0,033	-1,486
	85	358	0,00279	15,74	0,146	-0,835
	90	363	0,00275	6,55	0,352	-0,454
	95	368	0,00272	6,10	0,378	-0,423
Fosfato pH 8,0	75	348	0,00287	124,68	0,018	-1,734
	80	353	0,00283	93,30	0,025	-1,608
	85	358	0,00279	19,17	0,120	-0,920
	90	363	0,00275	7,37	0,312	-0,505
	95	368	0,00272	6,75	0,341	-0,467

Tabela 13. Coeficientes de inativação térmica (valores k), para *GFPuv* em tampão tris-HCl 10mM

Tampão	Temp. (°C)	Temp (K)	1/K	Valor D (min)	Valor k (min ⁻¹)	Log k
Tris - HCl pH 7,0	75	348	0,00287	14,97	0,154	-0,813
	80	353	0,00283	9,89	0,233	-0,633
	85	358	0,00279	5,01	0,460	-0,338
	90	363	0,00275	2,94	0,783	-0,106
	95	368	0,00272	1,72	1,339	0,127
Tris - HCl pH 7,5	75	348	0,00287	53,72	0,043	-1,368
	80	353	0,00283	23,65	0,097	-1,012
	85	358	0,00279	7,14	0,323	-0,491
	90	363	0,00275	4,22	0,546	-0,263
	95	368	0,00272	3,03	0,760	-0,119
Tris - HCl PH 8,0	80	353	0,00283	37,40	0,062	-1,211
	85	358	0,00279	13,69	0,168	-0,774
	90	363	0,00275	5,51	0,418	-0,379
	95	368	0,00272	4,33	0,532	-0,274
Tris - HCl pH 8,5	80	353	0,00283	51,16	0,045	-1,347
	85	358	0,00279	20,24	0,114	-0,944
	90	363	0,00275	6,79	0,339	-0,470
	95	368	0,00272	5,64	0,408	-0,389

Foram selecionadas representações gráficas relacionando $\text{Log}_{10} k$ e o inverso da temperatura absoluta ($1/K$), para cada solução-tampão estudada (figuras 41, 42, 43, 44 e 45).

Figura 41. Correlação entre Log₁₀ k e o inverso das temperaturas absolutas para o sistema da *GFPuv* em solução-tampão acetato 10mM, pH 6,0

Figura 42. Correlação entre Log₁₀ k e o inverso das temperaturas absolutas para o sistema da *GFPuv* em solução-tampão fosfato 10mM, pH 6,5

Figura 43. Correlação entre $\text{Log}_{10} k$ e o inverso das temperaturas absolutas para o sistema da GFPuv em solução-tampão tris-HCl 10mM, pH 7,5

Figura 44. Correlação entre $\text{Log}_{10} k$ e o inverso das temperaturas absolutas para o sistema da GFPuv em solução-tampão tris-HCl 10mM, pH 8,0

Figura 45. Correlação entre $\text{Log}_{10} k$ e o inverso das temperaturas absolutas para o sistema da *GFPuv* em solução-tampão tris-HCl 10mM, pH 8,5

Tabela 14. Valores de Energias de Ativação (E_a , kcal/mol), para os sistemas de *GFPuv* em soluções-tampão, calculados a partir das equações de reta das relações entre $\text{Log}_{10} k$ versus o inverso da temperatura absoluta ($1/K$)

tampão	pH	Equação $\text{Log}_{10} k$	R^2	E_a (kcal/mol)
Acetato	6,0	$= -4211,2(1/K) + 11,171$	0,89	19,27
	6,5	$= -5496,2(1/K) + 14,761$	0,91	25,15
	7,0	$= -6284,2(1/K) + 16,876$	0,92	28,76
Fosfato	6,5	$= -5720,2(1/K) + 15,315$	0,91	26,18
	7,0	$= -7708,9(1/K) + 20,696$	0,91	35,28
	7,5	$= -9316,1(1/K) + 25,049$	0,87	42,63
	8,0	$= -9325,3(1/K) + 25,012$	0,92	42,67
Tris-HCl	7,0	$= -6160,5(1/K) + 16,862$	0,995	28,19
	7,5	$= -8337,9(1/K) + 22,649$	0,96	38,15
	8,0	$= -8348,2(1/K) + 22,503$	0,94	38,20
	8,5	$= -8718,6(1/K) + 23,403$	0,94	39,90

Tabela 15. Valores z, valores de Energia de Ativação (Ea) para os sistemas de *GFPuv* em soluções-tampão acetato, fosfato e tris-HCl

pH	Acetato			Fosfato			Tris-HCl		
	Valor z (°C)	Q ₁₀	Ea (kcal/mol)	Valor z (°C)	Q ₁₀	Ea (kcal/mol)	Valor z (°C)	Q ₁₀	Ea (kcal/mol)
6,0	30,96	2,10	19,27						
6,5	23,42	2,67	25,15	22,42	2,79	26,18			
7,0	20,45	3,08	28,76	16,64	3,99	35,28	20,79	3,03	28,19
7,5				14,01	5,17	42,63	15,41	4,46	38,15
8,0				13,75	5,34	42,67	15,6	4,38	38,2
8,5							14,95	4,67	39,9

De acordo com os valores relacionados na tabela 15, o sistema *GFPuv* em tampão acetato, ao valor de pH 6,0, mostrou-se termoestável, apresentando valor z de 30,96°C e Energia de ativação (E_a) de 19,27 kcal/mol. Em relação ao valor de pH 6,50, os sistemas *GFPuv* em tampão acetato e fosfato mostraram termoestabilidades próximas, apresentando valores z de 23,42°C e 22,42°C, e valores de E_a de 25,15 kcal/mol e 26,18 kcal/mol, respectivamente. O tampão acetato ao valor de pH 6,0 conferiu maior termoestabilidade à proteína *GFPuv*, para a faixa de pH estudada.

Para os sistemas de *GFPuv* em soluções-tampão acetato e tris-HCl, ao valor de pH 7,0, a termoestabilidade da proteína mostrou-se equivalente, quanto aos valores z de 20,45°C e 20,79°C e valores de energia de ativação de 28,76 kcal/mol e 28,19 kcal/mol, respectivamente. Entretanto, em tampão fosfato (pH 7,0), a termoestabilidade da *GFPuv* foi 20% menor, sendo valor z de 16,64°C e valor E_a de 35,28 kcal/mol.

A termoestabilidade da proteína foi similar para os sistemas: (i) solução-tampão acetato aos valores de pH 6,5 e 7,0, e respectivos valores z de 23,42°C e 20,45°C; (ii) solução-tampão fosfato aos valores de pH 7,0, 7,5 e 8,0, e respectivos valores z de 16,64°C, 14,01°C e 13,75°C; (iii) solução-tampão tris-HCl aos valores de pH 7,5, 8,0 e 8,5 e respectivos valores z de 15,41°C, 15,6°C e 14,95°C.

A termoestabilidade da proteína foi maior em tampão acetato pH 6,0 (E_a = 19,27 kcal/mol), e foi equivalente para os sistemas formados por tampão acetato aos valores de pH 6,5 e 7,0 (E_a = 25,15 kcal/mol e 28,76 kcal/mol, respectivamente), tampão fosfato ao valor de pH 6,5 (E_a = 26,18 kcal/mol) e, em tampão tris-HCl ao valor de pH 7,0 (E_a = 28,19 kcal/mol).

Os sistemas de *GFPuv* em tampão fosfato aos valores de pH 7,5 e 8,0 (E_a = 42,63 kcal/mol e 42,67 kcal/mol) e em tampão tris-HCl aos valores de pH 8,0 e 8,5 (E_a = 38,2 kcal/mol e 39,9 kcal/mol) mostraram menor estabilidade térmica. Em tampão fosfato, o valor Q_{10} de 5,34 mostrou maior variação do valor D para a *GFPuv* em relação ao intervalo de temperatura

estudado, confirmando instabilidade térmica para este sistema em relação às condições estudadas.

Os dados apresentados confirmam a estabilidade da *GFPuv* estudada em trabalho anterior (Anexo 8), onde, a *GFPuv* acondicionada em solução-tampão tris-HCl, apresentou valor z de 12,87°C e valor E_a de 45,5 kcal/mol, para o mesmo intervalo de temperatura (75°C a 95°C), valores similares à estabilidade da *GFPuv* em solução-tampão fosfato, pH 8,0, de valor z de 13,75°C e valor E_a de 42,67 kcal/mol.

FORSYTH (1999) estudou isoenzimas da peroxidase, à temperatura de 75°C os valores D variaram de 0,01min a 7,38 min para a enzima nativa e de 0,096 min a 18,96 min para a enzima parcialmente inativada. A *GFPuv*, em pH 8,0, apresentou maior termoestabilidade quando submetida a tratamento térmico à temperatura de 75°C ($D_{pH\ 7,84} = 166,66$ min, em tampão fosfato e, $D_{pH\ 8,05} = 83,33$ min em tampão tris-HCl).

Em estudo da termoestabilidade de enzimas encontradas no leite, GRIFFITHS (1986), sugeriu o valor z de 8,0°C para a inativação da lactoperoxidase, no intervalo de temperatura entre 74°C e 83°C e, valor z de 15°C para a inativação de lisozima em intervalo de temperatura entre 61°C e 100°C. A menor termoestabilidade foi encontrada para a *GFPuv* em tampão tris-HCl, ao valor de pH 8,5, apresentando valor z de 14,95°C.

A termoestabilidade da lisozima em cerveja (MAKKI, 1996), apresentou em sistema contendo 15% de glicose aos valores de pH 5,20 e 7,20, valores z de 13,8°C e 14,1°C e valores de E_a de 42,0 kcal/mol e 41,1 kcal/mol, respectivamente e, em sistema contendo NaCl 1,0M ao pH 7,20, valor z 13,9°C e valor de E_a de 41,8 kcal/mol, resultados semelhantes aqueles obtidos para os sistema da *GFPuv* em tampão fosfato aos valores de pH 7,5 e 8,0.

Para a inativação térmica de *B. cereus* ATCC 7004 no intervalo de temperatura entre 92° - 106°C, MAZAS *et al.* (1999) sugeriu: (i) valor z de 8,3°C para o sistema ao valor de pH de 7,60 e atividade de água (A_a) 1,00, sem a adição de sais ao meio; (ii) valor z de 10,3°C para o sistema ao valor de pH 8,35, em solução 5,6M NaCl e atividade de água (A_a) 0,75. Para os

dois sistemas o valor z caracterizado para esporos de *B. cereus* foi inferior àqueles apresentados para a *GFPuv* nas condições estudadas.

A estabilidade térmica apresentada pela *GFPuv* em ampla faixa de pH (de 5,5 a 8,5) e em diferentes composições de soluções possibilita sua aplicação como indicador biológico em diversas situações.

A proteína *GFPuv* apresenta grande potencialidade para ser utilizada como indicador biológico em processos de branqueamento, pasteurização, desinfecção e de esterilização que utilizam vapor às temperaturas inferiores a 100°C.

CONCLUSÕES

Em sistema de tampão acetato a estabilidade da *GFPuv* expressa em termos de valores D (a 75°C), mostrou correlação linear para os valores de pH $\geq 5,50$.

Em sistema de tampão fosfato, a linearidade da relação entre os valores D e o aumento do valor de pH foi entre pH 6,19 e pH 7,50. Para valores de pH $\geq 7,50$, a estabilidade da proteína independeu do valor de pH da solução-tampão fosfato.

Em sistema de tampão tris-HCl 10 mM, observou-se linearidade dos valores D e valores de pH para o intervalo de pH entre 6,91 e 8,50. Para valores de pH $> 8,50$, verificou-se inconstância de relação entre os valores D obtidos experimentalmente e o aumento gradual do valor de pH da solução-tampão tris-HCl.

O tampão acetato ao valor de pH 6,0 conferiu maior termoestabilidade à proteína *GFPuv*, para a faixa de pH estudada.

Para os sistemas de *GFPuv* em soluções-tampão acetato e tris-HCl, ao valor de pH 7,0, a termoestabilidade da proteína mostrou-se equivalente.

Os sistemas de *GFPuv* em tampão fosfato aos valores de pH 7,5 e 8,0 e em tampão tris-HCl aos valores de pH 8,0 e 8,5 mostraram menor estabilidade térmica.

A proteína *GFPuv* apresenta grande potencialidade para ser utilizada como indicador biológico em processos que utilizam calor às temperaturas inferiores a 100°C.

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Anexo 01

Decaimento da concentração de *GFPuv* ($\mu\text{g GFPuv /mL}$) em diferentes soluções-tampão.

Temperatura de Tratamento - 75°C

Figura 01. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 4,98

Figura 02. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 5,18

Figura 03. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 5,32

Figura 04. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 5,50

Figura 05. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 5,66

Figura 06. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 5,67

Figura 07. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 5,90

Figura 08. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 6,13

Figura 09. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 6,19

Figura 10. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 6,19

Figura 11. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 6,28

Figura 12. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 6,40

Figura 13. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 6,49

Figura 14. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 6,50

Figura 15. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 6,67

Figura 16. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 6,81

Figura 17. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 6,89

Figura 18. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 6,91

Figura 19. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 7,01

Figura 20. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,11

Figura 21. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 7,14

Figura 22. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,20

Figura 23. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,34

Figura 24. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,36

Figura 25. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 7,41

Figura 26. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,50

Figura 27. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,60

Figura 28. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 7,61

Figura 29. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 7,67

Figura 30. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,74

Figura 31. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,84

Figura 32. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,05

Figura 33. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,50

Figura 34. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,65

Figura 35. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,90

Temperatura de Tratamento - 80°C

Figura 36. Decaimento da concentração de *GFPuv* em solução-tampão acetato, pH 5,18

Figura 37. Decaimento da concentração de *GFPuv* em solução-tampão acetato, pH 5,64

Figura 38. Decaimento da concentração de *GFPuv* em solução-tampão fosfato, pH 5,97

Figura 39. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 6,15

Figura 40. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 6,20

Figura 41. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 6,22

Figura 42. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 6,53

Figura 43. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 6,68

Figura 44. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 6,92

Figura 45. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 7,03

Figura 46. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,13

Figura 47. Decaimento da concentração de GFPuv em solução-tampão tris HCl, pH 7,50

Figura 48. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,55

Figura 49. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,91

Figura 50. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,08

Figura 51. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,45

Figura 52. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,84

Temperatura de Tratamento - 85°C

Figura 53. Decaimento da concentração de *GFPuv* em solução-tampão acetato, pH 4,91

Figura 54. Decaimento da concentração de *GFPuv* em solução-tampão acetato, pH 5,18

Figura 55. Decaimento da concentração de *GFPuv* em solução-tampão acetato, pH 5,32

Figura 56. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 5,63

Figura 57. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 5,64

Figura 58. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 5,92

Figura 59. Decaimento da concentração de *GFPuv* em solução-tampão acetato, pH 6,15

Figura 60. Decaimento da concentração de *GFPuv* em solução-tampão fosfato pH 6,20

Figura 61. Decaimento da concentração de *GFPuv* em solução-tampão fosfato, pH 6,50

Figura 62. Decaimento da concentração de GFP_{uv} em solução-tampão acetato, pH 6,53

Figura 63. Decaimento da concentração de GFP_{uv} em solução-tampão fosfato, pH 6,83

Figura 64. Decaimento da concentração de GFP_{uv} em solução-tampão acetato, pH 6,92

Figura 65. Decaimento da concentração de GFP_{uv} em solução-tampão acetato, pH 7,03

Figura 66. Decaimento da concentração de GFP_{uv} em solução-tampão fosfato, pH 7,27

Figura 67. Decaimento da concentração de GFP_{uv} em solução-tampão tris-HCl, pH 7,41

Figura 68. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 7,61

Figura 69. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,64

Figura 70. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,80

Figura 71. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,84

Figura 72. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,05

Figura 73. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,50

Figura 74. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,65

Figura 75. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,90

Temperatura de Tratamento - 90°C

Figura 76. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 5,19

Figura 77. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 5,72

Figura 78. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 6,03

Figura 79. Decaimento da concentração de *GFPuv* em solução-tampão fosfato, pH 6,19

Figura 80. Decaimento da concentração de *GFPuv* em solução-tampão acetato, pH 6,25

Figura 81. Decaimento da concentração de *GFPuv* em solução-tampão fosfato, pH 6,42

Figura 82. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 6,74

Figura 83. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 6,94

Figura 84. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 6,96

Figura 85. Decaimento da concentração de GFP_{uv} em solução-tampão acetato, pH 7,01

Figura 86. Decaimento da concentração de GFP_{uv} em solução-tampão tris-HCl, pH 7,27

Figura 87. Decaimento da concentração de GFP_{uv} em solução-tampão tris-HCl, pH 7,42

Figura 88. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,43

Figura 89. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 7,50

Figura 90. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 7,72

Figura 91. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 7,74

Figura 92. Decaimento da concentração de *GFPuv* em solução-tampão fosfato, pH 7,80

Figura 93. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 8,05

Figura 94. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 8,05

Figura 95. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 8,37

Figura 96. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 8,38

Figura 97. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,60

Figura 98. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,64

Figura 99. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,82

Figura 100. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 8,92

Figura 101. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 9,03

Temperatura de Tratamento - 95°C

Figura 102. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 5,11

Figura 103. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 5,19

Figura 104. Decaimento da concentração de GFPuv em solução-tampão acetato, pH 5,46

Figura 105. Decaimento da concentração de *GFPuv* em solução-tampão acetato, pH 5,72

Figura 106. Decaimento da concentração de *GFPuv* em solução-tampão fosfato, pH 5,89

Figura 107. Decaimento da concentração de *GFPuv* em solução-tampão fosfato, pH 6,14

Figura 108. Decaimento da concentração de *GFPuv* em solução-tampão fosfato, pH 6,19

Figura 109. Decaimento da concentração de *GFPuv* em solução-tampão acetato, pH 6,25

Figura 110. Decaimento da concentração de *GFPuv* em solução-tampão fosfato, pH 6,52

Figura 111. Decaimento da concentração de *GFPuv* em solução-tampão acetato, pH 6,74

Figura 112. Decaimento da concentração de *GFPuv* em solução-tampão acetato, pH 6,94

Figura 113. Decaimento da concentração de *GFPuv* em solução-tampão acetato, pH 7,01

Figura 114. Decaimento da concentração de *GFPuv* em solução-tampão fosfato, pH 7,05

Figura 115. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 7,08

Figura 116. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 7,11

Figura 117. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 7,34

Figura 118. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 7,44

Figura 119. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 7,44

Figura 120. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 7,68

Figura 121. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 7,72

Figura 122. Decaimento da concentração de *GFPuv* em solução-tampão fosfato, pH 7,82

Figura 123. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 7,89

Figura 124. Decaimento da concentração de *GFPuv* em solução-tampão fosfato, pH 7,95

Figura 125. Decaimento da concentração de *GFPuv* em solução-tampão fosfato, pH 8,0

Figura 126. Decaimento da concentração de GFPuv em solução-tampão fosfato, pH 8,0

Figura 127. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,05

Figura 128. Decaimento da concentração de GFPuv em solução-tampão tris-HCl, pH 8,34

Figura 129. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 8,38

Figura 130. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 8,55

Figura 131. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 8,63

Figura 132. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 8,70

Figura 133. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 8,92

Figura 134. Decaimento da concentração de *GFPuv* em solução-tampão tris-HCl, pH 8,97

Anexo 02

Representação Gráfica dos valores D (min) da *GFPuv* em
soluções-tampão para diferentes valores de pH

Figura 01. Valores D (min) da *GFPuv* em solução-tampão acetato 10mM para diferentes valores de pH, à temperatura de 80°C

Figura 02. Valores D (min) da *GFPuv* em solução-tampão acetato 10mM para diferentes valores de pH, à temperatura de 85°C

Figura 03. Valores D (min) da *GFPuv* em solução-tampão acetato 10mM para diferentes valores de pH, à temperatura de 90°C

Figura 04. Valores D (min) da *GFPuv* em solução-tampão acetato 10mM para diferentes valores de pH, à temperatura de 95°C

Figura 05. Valores D (min) da *GFPuv* em solução-tampão fosfato 10mM para diferentes valores de pH, à temperatura de 80°C

Figura 06. Valores D (min) da *GFPuv* em solução-tampão fosfato 10mM para diferentes valores de pH, à temperatura de 85°C

Figura 07. Valores D (min) da *GFPuv* em solução-tampão fosfato 10mM para diferentes valores de pH, à temperatura de 90°C

Figura 08. Valores D (min) da *GFPuv* em solução-tampão fosfato 10mM para diferentes valores de pH, à temperatura de 95°C

Figura 09. Valores D (min) da *GFPuv* em solução-tampão tris-HCl 10mM para diferentes valores de pH, à temperatura de 80°C

Figura 10. Valores D (min) da *GFPuv* em solução-tampão tris-HCl 10mM para diferentes valores de pH, à temperatura de 85°C

Figura 11. Valores D (min) da *GFPuv* em solução-tampão tris-HCl 10mM para diferentes valores de pH, à temperatura de 90°C

Figura 12. Valores D (min) da *GFPuv* em solução-tampão tris-HCl 10mM para diferentes valores de pH, à temperatura de 95°C

Anexo 03

Valor z

Correlação entre Log_{10} Valores D (min) e temperaturas de tratamento para *GFPuv* em diferentes soluções-tampão.

Figura 01. Correlação entre Log₁₀ Valores D (min) e temperaturas de tratamento para *GFPuv* em solução-tampão acetato 10mM, pH 6,5

Figura 02. Correlação entre Log₁₀ Valores D (min) e temperaturas de tratamento para *GFPuv* em solução-tampão acetato 10mM, pH 7,0

Figura 03. Correlação entre Log₁₀ Valores D (min) e temperaturas de tratamento para *GFPuv* em solução-tampão fosfato 10mM, pH 7,0

Figura 04. Correlação entre Log₁₀ Valores D (min) e temperaturas de tratamento para *GFPuv* em solução-tampão fosfato 10mM, pH 7,5

Figura 05. Correlação entre Log₁₀ Valores D (min) e temperaturas de tratamento para *GFPuv* em solução-tampão fosfato 10mM, pH 8,0

Figura 06. Correlação entre Log₁₀ Valores D (min) e temperaturas de tratamento para *GFPuv* em solução-tampão tris-HCl 10mM, pH 7,0

Anexo 04

Energia de Ativação

Correlação entre $\text{Log}_{10} k$ e o inverso das temperaturas absolutas
para o sistema da *GFPuv* em soluções-tampão

Figura 01. Correlação entre $\text{Log}_{10} k$ e o inverso das temperaturas absolutas de tratamento para o sistema da *GFPuv* em solução-tampão acetato 10mM, pH 6,5

Figura 02. Correlação entre $\text{Log}_{10} k$ e o inverso das temperaturas absolutas de tratamento para o sistema da *GFPuv* em solução-tampão acetato 10mM, pH 7,0

Figura 03. Correlação entre $\text{Log}_{10} k$ e o inverso das temperaturas absolutas de tratamento para o sistema da *GFPuv* em solução-tampão fosfato 10mM, pH 7,0

Figura 04. Correlação entre $\text{Log}_{10} k$ e o inverso das temperaturas absolutas de tratamento para o sistema da *GFPuv* em solução-tampão fosfato 10mM, pH 7,5

Figura 05. Correlação entre $\text{Log}_{10} k$ e o inverso das temperaturas absolutas de tratamento para o sistema da GFPuv em solução-tampão fosfato 10mM, pH 8,0

Figura 06. Correlação entre $\text{Log}_{10} k$ e o inverso das temperaturas absolutas de tratamento para o sistema da GFPuv em solução-tampão Tris-HCl 10mM, pH 7,0

Anexo 05

“Selective Permeation and Organic Extraction of Recombinant Green Fluorescent Protein (GFP_{uv}) from *Escherichia coli*”

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Objetivo

Este trabalho visou o estudo da permeação/extração da proteína verde fluorescente (GFP_{uv}) das células de *E. coli* DH5- α por dois métodos distintos:

- pelo método físico de congelamento e descongelamento associado ou não a sonicação seguido da purificação por partição em três fases;
- pelo método químico de partição por 3 fases – *three phase partitioning* (TPP), unicamente.

Conclusões

A permeação seletiva de GFP_{uv} realizada pelo método de congelamento e descongelamento associado a sonicação foi equivalente à quantidade de proteína extraída das células pelo processo direto de partição em três fases. Embora os métodos tenham se mostrado equivalentes o primeiro método, apresentou melhores resultados após a purificação das amostras por coluna cromatográfica de interação hidrofóbica.

Research article

Selective Permeation and Organic Extraction of Recombinant Green Fluorescent Protein (*gfp_{uv}*) from *Escherichia coli*

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Abstract

Background: Transformed cells of *Escherichia coli* DH5- α with pGFPuv, induced by IPTG (isopropyl- β -D-thiogalactopyranoside), express the green fluorescent protein (*gfp_{uv}*) during growth phases. *E. coli* subjected to the combination of selective permeation by freezing/thawing/sonication cycles followed by the three-phase partitioning extraction (TPP) method were compared to the direct application of TPP to the same culture of *E. coli* on releasing *gfp_{uv}* from the over-expressing cells.

Material and Methods: Cultures (37°C/100 rpm/ 24 h; $\mu = 0.99 \text{ h}^{-1} - 1.10 \text{ h}^{-1}$) of transformed (pGFP) *Escherichia coli* DH5- α , expressing the green fluorescent protein (*gfp_{uv}*, absorbance at 394 nm and emission at 509 nm) were sonicated in successive intervals of sonication (25 vibrations/pulse) to determine the maximum amount of *gfp_{uv}* released from the cells. For selective permeation, the transformed previously frozen (-75°C) cells were subjected to three freeze/thaw (-20°C/ 0.83°C/min) cycles interlaid by sonication (3 pulses/ 6 seconds/ 25 vibrations). The intracellular permeate with *gfp_{uv}* in extraction buffer (TE) solution (25 mM Tris-HCl, pH 8.0, 1 mM β -mercaptoethanol β -ME, 0.1 mM PMSF) was subjected to the three-phase partitioning (TPP) method with *t*-butanol and 1.6 M ammonium sulfate. Sonication efficiency was verified on the application to the cells previously treated by the TPP method. The intra-cell releases were mixed and eluted through methyl HIC column with a buffer solution (10 mM Tris-HCl, 10 mM EDTA, pH 8.0).

Results: The sonication maximum released amount obtained from the cells was 327.67 $\mu\text{g gfp}_{uv}/\text{mL}$ (20.73 $\mu\text{g gfp}_{uv}/\text{mg}$ total proteins – BSA), after 9 min of treatment. Through the selective permeation by three repeated freezing/thawing/sonication cycles applied to the cells, a close content of 241.19 $\mu\text{g gfp}_{uv}/\text{mL}$ (29.74 $\mu\text{g gfp}_{uv}/\text{mg}$ BSA) was obtained. The specific mass range of *gfp_{uv}* released from the same cultures, by the three-phase partitioning (TPP) method, in relation to total proteins, was higher, between 107.28 $\mu\text{g}/\text{mg}$ and 135.10 $\mu\text{g}/\text{mg}$.

Conclusions: The selective permeation of *gfp_{uv}* by freezing/thawing/sonication followed by TPP separation method was equivalent to the amount of *gfp_{uv}* extracted from the cells directly by TPP; although selective permeation extracts showed better elution through the HIC column.

Background

The fluorescent green protein, *gfp* extracted from *Aequorea victoria* shows a maximum excitation under ultraviolet light (UV) in the interval of $\lambda = 395\text{--}498\text{ nm}$ and emission in the interval of $\lambda = 490\text{--}520\text{ nm}$, with the maximum peak at $\lambda = 508\text{--}509\text{ nm}$. The recombinant fluorescent green protein, *gfp_{uv}*, expressed by transformed (*pGFP*, Clontech) cells of *Escherichia coli*, was developed by introducing point mutations in an *in vitro* wild *gfp* DNA, replacing three amino acids (Phe⁹⁹ for Ser; Met¹⁵³ for Thr; and Val¹⁶³ for Ala, based on the amino acid numbering of wild *gfp*). The resulting *gfp_{uv}* is expressed two to three times faster in *E. coli* strains and shows 18 times brighter fluorescence than the native *gfp*; the maximum peaks for fluorophore excitation being at 395 nm and centered at 508–509 nm for emission [1].

The recombinant *gfp_{uv}* is a globular protein, compact and acidic, made up of 27 kDa monomers (with 238 amino acids), having a propensity to dimerize. The protein fluorescence is stable between pH 5.5 and 11.5, with the optimum at pH 8.0.

The first step for the *gfp_{uv}* purification process was the intracellular release and isolation of the protein into an aqueous extraction buffer – TE (25 mM Tris-HCl, pH 8.0, 1 mM β -mercaptoethanol, " β -ME", 0.1 mM phenylmethylsulfonylfluoride, "PMSF"). During extraction procedures, the proteolysis of the C-terminal extremity of *gfp_{uv}* was inhibited by working at cool temperatures (0°C to 4°C), and in the presence of PMSF. The addition of β -ME and the maintenance of pH at 8.0 enhances protein stability [1].

Several methods are used currently for cellular partial (permeation) or total cellular disruption. For sake of convenience, they may be classified as (i) physical mechanical (ultra sonication, bead mill, blade and high-pressure homogenizers) and non-mechanical treatments (thermal shock, freeze and thaw); and a combination (freezing/thawing cycles interlaid with sonication); (ii) chemical methods for selective protein fractionation by the use of (a) organic solvent (alcohol, toluene, acetone); (b) inorganic acids and bases (pH variation) and salts (salting in and salting out phenomena); (c) combination of salting out (ammonium sulfate) and cosolvent (ethanol, t-butanol); (d) organic water soluble polymers (polyethylene glycol "PEG" molecular weight 4,000 or greater); (iii) affinity methods (chromatography-HIC: hydrophobic interaction chromatography; electrophoresis; affinity phase partitioning); (iv) biological methods with enzyme digestion of microbial membranes (lysozyme); (v) combination of those techniques (physical permeation plus chemical cellular disruption followed by affinity method).

During the isolation procedures from the bacterial cells, the stability of the required protein structure is of fundamental importance. Through the fractionation technique employed as the first step, the maintenance of the protein structure and activity is more important than the purity attained. A protocol selected to release the proteins from the intracellular compartments must provide the best recovery and purity, avoid proteolysis by the addition of protease inhibitors, and minimum binding to either particulates, high molecular weight proteins or non-protein substances that may prevent the recovery of the protein.

For the purpose of *gfp_{uv}* purification, Yakhnin et al [2] proposed a two-step procedure for the extraction of *gfp_{uv}* with ethanol into the organic phase and subsequent protein re-extraction into the aqueous phase, to reduce the loss of most of the *gfp_{uv}* during multiple chromatography steps. The ethanol extractions were made from the entire suspension in 70% ammonium sulfate $((\text{NH}_4)_2\text{SO}_4)$. The re-extraction in the aqueous $(\text{NH}_4)_2\text{SO}_4$ was performed with n-butanol added to the ethanol extract. The authors observed that one chromatography final step was enough to purify *gfp_{uv}* from nonprotein substances.

The addition of a polar miscible solvent, such as ethanol or acetone, to an aqueous extract containing proteins has a variety of effects that combined, lead to protein precipitation. The principal effect is the reduction in water activity. The solvating power of water for a charged, hydrophobic protein molecule is decreased as the concentration of organic solvent increases, which causes a bulk displacement of water plus the partial immobilization of water molecules through hydration of the organic solvent molecules. The most hydrophobic molecules or proteins may be soluble in 100% organic solvent [3].

The most advantageous salt for protein isolation and further purification, through fractionation methods, is ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$, which forms saline bridges with polar regions, stabilizing the molecule [2–8]. The main advantages of ammonium sulfate fractionation method, through salting out at high salt concentration (2–3 M ammonium sulfate), are (a) the stabilization of protein precipitate over long-term storage, (an easy way of packaging and commercialization); (b) the prevention of proteolysis and bacterial action and (c) the conservation of the protein precipitates, either before centrifuging or as a pellet, a good form in which a sample may be kept for further purification steps [3].

The addition of t-butanol to the ammonium sulfate solution of proteins consolidates the technique known as three-phase-partitioning (TPP), which is characterized by three distinct phases: an upper t-butanol, a lower aqueous

phase containing $(\text{NH}_4)_2\text{SO}_4$ at high concentration, and a middle layer [4–7]. The mixing of the upper phase with lower phase creates a third intermediate phase of precipitate material. The concentration of the required protein in the third phase depends on the $(\text{NH}_4)_2\text{SO}_4$ concentration. t-butanol stabilizes protein structure, inhibits proteases and protein/protein interactions [4,5]. These are similar qualities shown by ammonium sulfate during the salting out phenomenon.

$(\text{NH}_4)_2\text{SO}_4$ at high concentration promotes hydration of polar regions and dehydration of the hydrophobic regions on the protein surface, without direct interaction to molecular structure. Salting out depends on hydrophobic regions on the surface of proteins, where solubility is strongly reduced. At high salt concentration, the salt ions become solvated easier than polar regions on the protein surface. Furthermore, the freely available water molecules become scarce and the bare polar regions that have their water solubility reduced tend to aggregate and form clusters of different sizes [2,3,5].

In contrast, proteins and particulate material with a majority of non-polar surface residues may remain in solution even at the higher salt concentration. With the TPP method, those residues in contact with t-butanol are precipitated and form the first fraction of a floating intermediate phase, characterized by white cluster that is difficult to re-dissolve even in extraction buffer (TE). For the TPP method, the first white middle layer is a collection of high molecular weight contaminants and debris, precipitating at up to 25% ammonium sulfate saturation with conventional salting out fractionation method.

The binding of t-butanol to the hydrophobic regions of the protein molecules in the homogenate depends on the both the concentrations of the $(\text{NH}_4)_2\text{SO}_4$ and the required protein, which floats into the third phase at the TPP technique. At that time, when t-butanol is added a second time to the homogenate in the presence of a high concentration of ammonium sulfate, a protein is precipitated as an interface between the organic and aqueous phases. Therefore, the t-butanol stabilizes protein aggregates in the middle layer at the second TPP, preventing protein/protein interaction [5]. The thin middle layer is easily dissolved in the extraction buffer (TE, with added EDTA) and may be stored (4°C) for latter purification steps or subjected to further fractionation method by hydrophobic interaction chromatography (HIC – affinity chromatography).

TPP that is an emerging bioseparation technique presents the advantages of conventional salting out, isoionic precipitation, co-solvent precipitation and osmolytic and ko-

smotropic precipitation of proteins. It is easily scalable and can be used directly with crude suspensions [6,7].

Consequently, TPP was the technique chosen for direct fractionation of *gfp_{uv}*.

In this study, the bacteria cellular release was blended in 450 µl of TE (25 mM Tris-HCl, β-ME, PMSF). The homogenate was mixed with 300 µL of 4 M ammonium sulfate and 750 µL of t-butanol (ratio 1:1) was added (Ward personal communication) [8]. TPP was performed at room temperature. The sample was vigorously stirred, centrifuged, and the three phases formed were collected separately. The large amounts of *gfp_{uv}* present in the lower aqueous layer (containing $(\text{NH}_4)_2\text{SO}_4$), was subjected to TPP several times. The intermediate middle layer containing *gfp_{uv}* was precipitated. The precipitated *gfp_{uv}* was collected and re-suspended in extraction buffer (TE, with added EDTA) and then fractionated on to the HIC column.

Sharma & Gupta [6] employed the TPP method for direct one step purification of protein inhibitor from the crude wheat germ extract. The authors observed best results (25-fold purification with an activity recovery of 85%) with a 1:1 ratio of t-butanol to crude extract and 30% ammonium sulfate (w/v) at 20°C. Sharma & Gupta [7] purified phospholipase from *Daucus carota* by a single step of the TPP procedure and obtained 13-fold purification with an activity of 72%.

The purpose of the research was to compare the association of selective permeation by freezing/ thawing/ sonication (FTS) cycles applied to the transformed cells of *E. coli* followed by the three-phase-partitioning extraction (TPP) method and the direct application of TPP to the same culture of *E. coli* on releasing *gfp_{uv}* from the over-expressing cells.

Material and methods

Transformation

The *Escherichia coli* DH5-α [2] were transformed with pGFPuv (Clontech, CA, USA) [9], by the standard calcium chloride method [5]. The transformed cells of *Escherichia coli* DH5-α with pGFPuv (Clontech, CA, USA) [2,5] were stored at (-75°C) into LB/amp broth with glycerol added in the proportion of 1:1.

Expression

A 24 h (37°C/100 rpm) culture of *E. coli* was developed in 25 mL Luria Bertani (LB; Difco, USB/Mexico, OH, USA) medium, supplemented with ampicillin (100 µg/mL Boehringer, Mannheim, Germany). When the broth cultures attained a $\text{OD}_{660\text{nm}} = 0.7\text{--}0.8$ (10^8 CFU/mL) isopropyl-β-D-thiogalactopyranoside (IPTG; dioxane free, USD/Italy,

Figure 1
Pelleted cells of *E. coli*, expressing green fluorescent protein (*gfp_{uv}*).

OH, USA) was added to a final concentration of 0.5 mM [10], as inducer of *gfp_{uv}* expression by the mutant *gfp_{uv}* which is under tight control of the *lacZ* protein β -galactosidase promoter/repressor.

Growth conditions

The growth culture was centrifuged ($1000 \times g/30$ min/ 4°C), the supernatant was decanted, the pelleted cells were observed under UV light ($\lambda = 395$ nm) and resuspended in 1.0 mL cold extraction buffer solution (TE: 25 mM Tris-HCl, pH 8.0 – Trizma® Base, Sigma, MO, USA; 1.0 mM β -mercaptoethanol " β -ME", Pharmacia Biotech, Sweden; 0.1 mM phenylmethylsulfonylfluoride "PMSF", USB, Switzerland), dispersed and frozen at -75°C . The length of time the pelleted cells were held frozen was not monitored. (Ultra Freezer-Kelvinator Series 100 – Manitowoc, WI, USA). (Figure 1. The pelleted *E. coli* cells) The uniform dispersed suspension was thawed (4°C), subjected to blending and pelleted again by centrifugation.

The supernatant was read for total protein ($\text{OD}_{280\text{nm}}$) and isolated *gfp_{uv}* (absorbency = 395 nm; emission = 509 nm) released. The fluorescent pellet was resuspended and dispersed into 1.0 mL TE. Both, supernatant and pelleted cells, were stored at 4°C and processed further:

(i) *Sonication to the total permeation of gfp_{uv}*: A pelleted frozen (-75°C) 24 h culture of *E. coli* was dispersed into 20 mL of TE and sonicated (High Intensity Ultrasonic Processor, Vibram cells, model VC 100, Sonic & Materials, CT, USA). A 3 mm microtip ultrasonic processor was inserted in the tube of dispersed suspension (kept immersed in an ice-salt bath) and sonicated with 3, 6, 12, 24, 48, 96, 192,

384-fold pulses, over total intervals of 18s, 54s, 126s, 270s, 558s, 1134s, 1728s, 2880s. Each pulse was at 25 vibration amplitude at alternating cycles of 6 seconds on and 1.0 second off. Between sonication performed intervals, the suspension was centrifuged, a 1.0 mL aliquot of the supernatant was read for total protein and released *gfp_{uv}* (see Table 1).

Table 1: Sonication of over expressing cells of *E. coli*.

Time (seconds)	Total proteins (BSA) (mg/mL)	<i>gfp_{uv}</i> ($\mu\text{g/mL}$)	<i>gfp_{uv}</i> /BSA ($\mu\text{g/mg}$)
0	0.87	25.79	29.53
18	1.62	39.30	24.30
54	3.04	65.89	21.68
126	6.80	167.56	24.63
270	6.19	184.48	29.82
558	15.81	327.67	20.73
1134	9.83	222.68	22.64
1728	20.29	395.14	19.47
2880	14.05	268.28	19.10

(ii) *Three-Phase-Partitioning (TPP) extraction method* [4–8]. A pelleted frozen (-75°C) 24 h culture of *E. coli* was dispersed into 1.0 mL TE (4°C) and subjected to direct isolation by TPP. To each aliquot of 450 μL , 300 μL of 4 M $(\text{NH}_4)_2\text{SO}_4$ and 750 μL of *t*-butanol were added [6–8]. The mixture was vortexed for 1.0 min, allowed to settle for complete phase separation and centrifuged ($6,000 \times g/3$ min). The *t*-butanol upper layer and the white interfacial precipitate were removed and discarded. 750 μL of *t*-butanol was mixed into the lower aqueous layer. The mixture was allowed to settle and centrifuged. The upper layer was discarded. The interfacial green layer was collected and dissolved in 450 μL TE buffer solution and kept at 4°C for spectrophotometer and spectrofluorometer readings and partial purification through hydrophobic interaction chromatography (HIC). While the lower layer was still fluorescent, it was subjected to repeated TPP by mixing in 750 μL *t*-butanol, centrifugation and intermediate phase separation. (Figure 2)

(iii) *Freezing/Thawing/ Sonication (FTS) selective permeation of gfp_{uv}* [10]: A pelleted frozen (-75°C) 24 h culture of *E. coli* was dispersed into 1.0 mL TE. The re-suspended pellets were subjected to three repeated cycles of slow freezing (0.83°C/min until samples reached -20°C) and thawing (0.83°C/min , at room temperature), in a freezer-dryer (FTS System™, Secfroid, Lyolab G, NY, USA) chamber (Dura Stop™ MP). With PT-100 probes, inserted into the pelleted suspension, the freezing/thawing tempera-

Figure 2
The gfp_{uv} extracted by three phase partitioning ("TPP") method and dispersed in extraction buffer.

tures were registered every minute through a software "lyphoware" for Windows (Figure 3). The phenomenon of superfusion on the freezing of the samples was observed to happen between (-11°C) and (-14°C) , after which the samples reached (-20°C) at $0.83^{\circ}\text{C}/\text{min}$ [10]. Between the freezing/thawing cycles, the sample was subjected to three-fold pulse sonication in an ice-water bath over a total interval of 23s. Between the freezing/thawing/sonication cycle, cells were pelleted by centrifugation, and the supernatant was read for total protein and released gfp_{uv} (see Table 03).

(iv) *Three-Phase-Partitioning (TPP) extraction method associated to the FTS procedure.* The pellets from the 3rd FTS cycle were re-suspended into 450 μL of TE and subjected to the TPP extraction method.

(v) *Direct extraction by the TPP method.* Each pelleted frozen (-75°C) 24 h culture (I & J) of *E. coli* was dispersed into 1.0 mL of TE and vortexed for total uniform dispersion. The suspension was divided into two groups, as follows:

(a) A 450 μL sample of each culture was subjected to: (i) direct 2-step extraction by the TPP method; (ii) followed by sonication procedure (3-fold pulse/ 25 vibration amplitude/ alternating cycles of 6 seconds on and 1.0 second off) in an ice-water bath for 23s and (iii) a final 1-step TPP extraction.

(b) The other 450 μL sample of each culture was previously subjected to: (i) the sonication procedure (three-fold 6s pulse at 25 vibration amplitude) in an ice-water bath for 23s; (ii) followed by one-step TPP extraction method.

(vi) *Fast Flow methyl hydrophobic interaction chromatography (HIC) for ion exchange separation and concentration of gfp_{uv} from the extraction mixtures.* Equal volumes (1:1) of the TE containing gfp_{uv} and 4 M $(\text{NH}_4)_2\text{SO}_4$ were mixed, blended and centrifuged. At room temperature, 1.0 mL of the clarified supernatant was loaded onto a methyl HIC column fast flow, pre-equilibrated with 2 M $(\text{NH}_4)_2\text{SO}_4$. gfp_{uv} was adsorbed at the top of the methyl hydrophobic matrix. The column was loaded with 250 μL of 1.3 M $(\text{NH}_4)_2\text{SO}_4$ which carried the gfp_{uv} throughout the column bed. The protein was eluted with 1.0 mL buffer solution (10 mM Tris-HCl, 10 mM EDTA, pH 8.0).

Concentrations

The supernatant was read for total protein (based on BSA) and isolated gfp_{uv} released. The fluorescent bright green intact cells with easily defined outlines were observed under an Olympus (BX-60-IV FLA System Attachment, NY, USA) microscope, through filters for the detection of fluorescent cells [U-MWG (510–550 nm); U-MWU (330–385 nm); U-MNV (400–410 nm)]. Recombinant green fluorescent protein (gfp_{uv}) absorbs at UV (330–385 nm) and blue (400–410 nm) emission, with a maximum absorbance peak at 395 nm and a minor at 470 nm, emitting maximum green light at 509 nm. The spectrum was confirmed through Fluorescence Spectrophotometer F-2000 – Hitachi (OH, USA) spectra.

Standard Curves

The fluorescence intensity of gfp_{uv} detected in a cleared bacterial cell lysate was measured using the Fluorescence Spectrophotometer, with an excitation filter of 394 nm and an emission filter of 505 nm. The fluorescence intensity of the experimental samples was compared to the standard curve (gfp_{uv} $\mu\text{g}/\text{mL} = 0.001 * (\text{fluorescence intensity}) - 0.1133$; $R^2 = 0.995$) to determine the amount of gfp_{uv} released from the cells. The standard curve was prepared using known amounts (between 6.66 $\mu\text{g}/\text{mL}$ and 0.59 $\mu\text{g}/\text{mL}$) of purified recombinant gfp_{uv} (Clontech) diluted in the same buffer solution (10 mM Tris-HCl, pH 8.0, 1.0 mM β ME, 0.1 mM PMSF).

The total protein released in the medium from *E. coli* cells was expressed in mg/mL and compared with total protein concentrations expressed as purified bovine serum albumin (BSA, mol wt. 66 kDa, Sigma, MO, USA) at $\lambda = 280$ nm in a spectrophotometer. The total protein concentrations in the buffer solution ranged from 100 to 1000 $\mu\text{g}/\text{mL}$, the maximum $\text{OD}_{280\text{nm}}$ being 0.615; and the comparative relationship between total proteins and BSA was made through the standard curve (total protein $\mu\text{g}/\text{mL} = 1727.2 * (\text{OD}_{280\text{nm}}) - 26.863$; $R^2 = 0.9943$).

Freeze/Thaw Cycles applied to *E. coli* cells

Figure 3
Freeze – Thaw cycles applied to *E. coli* cells.

Results

In Tables 1, 2 and 3, the results were expressed by total protein (BSA, mg/mL), released gfp_{uv} (μ g/mL) content, gfp_{uv} yield (%), μ g/ μ g) in relation to the total released gfp_{uv} content obtained; the specific gfp_{uv} mass being expressed by μ g of gfp_{uv} in relation to mg of total protein (μ g gfp_{uv} /mg BSA).

(i) Sonication of the cells to complete permeation and isolation of gfp_{uv} contents (Table 1)

Sonication was performed on cells of *E. coli* DH5- α expressing a high concentration of gfp_{uv} to verify the maximum protein content that can be released from the cells. After 558s sonication interval treatment, a maximum concentration of 327.67 μ g gfp_{uv} /mL was obtained, equivalent to the specific mass of 20.73 μ g gfp_{uv} /mg BSA.

It was interesting to observe that the values of specific mass had varied from 21.68 μ g/mg to 29.82 μ g/mg, indicating that during the sonication procedure the amount of released gfp_{uv} was proportional to the other proteins and molecules extracted from the sonicated cells.

(ii) Isolation by three-phase partitioning (TPP) method, using ammonium sulfate (final concentration 1.6 M) and t-butanol (Table 2)

In this work, the pelleted sample was diluted in 450 μ L of extraction buffer (TE: 25 mM Tris-HCl, pH 8.0, β -ME, PMSF). It was followed by addition of 300 μ L of 4 M $(NH_4)_2SO_4$ and, after that, 750 μ L t-butanol (ratio 1:1). The sample was stirred for 1.0 min, centrifuged at $10,000 \times g$ / 3 min and the three phases formed were collected separately. The first white middle layer (an white disc in-

Table 2: Three-phase-partitioning (TPP) method applied directly to cells of *E. coli* with specific growth rate constant (μ , h⁻¹): of 0.99 h⁻¹ for culture A, 1.05 h⁻¹ for cultures B, C, D, E and of 1.10 h⁻¹ for cultures F, G, H.

Sample	TPP (I)		
	<i>gfp_{uv}</i> (μ g/mL)	BSA (mg/mL)	<i>gfp_{uv}</i> /BSA (μ g/mg)
A1	354.08	3.30	107.28
A2	444.05	3.29	135.10
A (Pool)	378.53	3.98	95.11
B1	46.68	2.60	18.05
B2	38.59	2.24	17.26
B (Pool)	45.66	2.42	18.87
C	39.60	1.11	35.65
D	15.92	0.41	38.86
E	28.12	0.73	38.70
F1	15.78	0.52	30.42
F2	11.56	0.22	52.69
F (Pool)	5.47	0.67	8.12
G	19.42	0.79	24.53
H1	16.69	0.82	20.44
H2	9.16	0.19	47.67
H	4.78	0.67	7.09

terface) was a result of remaining high molecular weight contaminants and debris [4,5,8].

At the first stage, *gfp_{uv}* did not precipitate completely. Then, the lower aqueous layer was subjected to second addition of t-butanol and, at the separation of the three phases, the middle layer containing the precipitated *gfp_{uv}* was collected and dissolved into 500 μ L TE. The solution was eluted through a HIC (*Methyl Support Hydrophobic interaction Chromatography*) for partial purification.

The extraction through the TPP method applied to the 24 h culture A of high expression in *gfp_{uv}* fluorescence (Table 2), resulted in concentrations that varied from 354.08 to 444.05 μ g *gfp_{uv}*/mL, for a pool (extract mixture 1:1) of 378.53 μ g *gfp_{uv}*/mL, corresponding to specific masses (SM) of 107.28, 135.10, and 95.11 μ g *gfp_{uv}*/mg BSA. Even though the direct sonication of the cells (Table 1) and the direct application of the TPP method (Table 2) released similar concentration ranges of *gfp_{uv}*, the sonication caused a higher release of other molecules (BSA) that reduced the SM. Therefore, the specific mass for TPP was approximately five times higher than that obtained by the sonication procedure. Through the TPP method, proteins (other than *gfp_{uv}*), nucleic acids and other molecules were precipitated and separated from *gfp_{uv}*.

Cultures B, C, D and E showed (Table 2) expressions in μ g *gfp_{uv}*/mL and SM (μ g *gfp_{uv}*/mg BSA), respectively, 45.66 μ g/mL and 18.87 μ g/mg for culture B; 39.60 μ g/mL and 35.65 μ g/mg for culture C; 15.92 μ g/mL and 38.86 μ g/mg for culture D; 28.12 μ g/mL and 38.70 μ g/mg for culture E. The TPP method extracted lower amounts of contaminants for concentrations lower than 30 μ g *gfp_{uv}*/mL, when the specific mass gradually increased, as shown in cultures F, G and H. The TPP method not only extracted *gfp_{uv}* but also carried out a selective isolation of the protein.

For higher fluorescence (> 30 μ g *gfp_{uv}*/mL), the formed intermediate white ring proceeding from the contaminant precipitation was observed to entrap low amount of *gfp_{uv}*, which was lost. On the other hand, the TPP method cleaned the isolated *gfp_{uv}* solution, making subsequent elution through the HIC easier.

Cultures F, G and H showed (Table 2) half the expression of *gfp_{uv}*, as follows: 15.78 (F), 19.42 (G) and 16.69 (H) μ g *gfp_{uv}*/mL, versus 46.68 (B1), 38.59 (B2), 39.60 (C), 15.92 (D) and 28.12 (E) μ g *gfp_{uv}*/mL. However, the specific masses of these cultures B, C, D and E, F, G, H were equivalent, showing that lower concentrations of *gfp_{uv}* improved their isolation from the cells and contaminants.

Sharma [6] used the three-phase-partitioning (TPP) method to purify crude extract of protease/amylase inhibitors at 20°C; t-butanol was employed at a ratio of 1:1, and (NH₄)₂SO₄ at 30%; and the recovery was about 85% and 25-fold purification.

(iii) Permeation by physical procedures (freezing/thawing/sonication procedures) associated to the TPP method (Table 3)

The freezing (-75°C) permeated 69.85 μ g *gfp_{uv}*/mL (A1), five times less than the TPP method (354.08 μ g *gfp_{uv}*/mL, sample A₁, Table 2); however, the specific contents were similar, respectively 86.25 and 107.28 μ g *gfp_{uv}*/mg BSA. Therefore, slow freezing allowed a cleaner permeate than the extraction by TPP, which released four times more contaminants (3.30 mg BSA/mL) than permeation did (0.81 mg BSA/mL).

It was observed that, for culture A1 (Table 2), with a high concentration of *gfp_{uv}* expressed, the TPP method extracted between 354.08 and 444.05 μ g *gfp_{uv}*/mL from the cells, 70% more than the slow freezing (-75°C). This unit operation was followed by three repeated cycles of freezing/thawing/sonication (FTS), which caused a total permeation of 241.19 μ g *gfp_{uv}*/mL, distributed as follows (Table 3): 29% (69.85 μ g/mL) by freezing (-75°C), 24% (57.83 μ g/mL), 38% (91.78 μ g/mL), and 9% (21.73 μ g/mL) after the 1st, 2nd and 3rd FTS cycles, respectively. Penna et al. [10] verified that for increasing amounts of over-expressed

Permeation (FTS)

Permeation (FTS)																
Sample	(-75°C)			1° FTS			2° FTS			3° FTS			Pool	TPP		
	gF_{uv} μg/mL	BSA mg/mL	$\text{gF}_{uv}/\text{BSA}$ μg/mg	gF_{uv} μg/mL	BSA mg/mL	$\text{gF}_{uv}/\text{BSA}$ μg/mg	gF_{uv} μg/mL	BSA mg/mL	$\text{gF}_{uv}/\text{BSA}$ μg/mg	gF_{uv} μg/mL	BSA mg/mL	$\text{gF}_{uv}/\text{BSA}$ μg/mg		gF_{uv} μg/mL	BSA mg/mL	$\text{gF}_{uv}/\text{BSA}$ μg/mL
A1	69.85	0.81	86.25	57.83	2.80	20.62	91.78	3.89	23.61	21.73	0.61	35.87		44.49	1.10	40.32
A2	15.23	1.95	9.24				12.04	5.06	2.38	5.78	5.14	1.12		17.83	0.48	37.52
A3	2.95	0.20	14.81													
(Pool)	44.79	1.75	25.57	57.85	2.80	20.62	55.18	4.18	13.19	21.58	1.66	13.00	41.14	2.15	19.11	23.97
B	6.81	0.35	19.72	7.75	1.77	4.36	4.93	0.79	6.27	3.28	0.48	6.84	13.77	1.54	8.93	18.40
C	5.39	0.59	9.09	13.73	2.00	6.86	2.77	0.57	4.88	3.37	0.59	5.75	13.46	1.33	10.13	4.28
D	5.39	0.47	11.37	7.02	0.92	7.65	2.82	0.59	4.76	1.38	0.20	6.93	12.44	0.62	20.10	2.16
E	7.10	0.38	18.70	16.54	1.75	9.43	3.71	0.57	6.54	1.26	0.14	9.13	13.40	0.96	13.97	2.14
F	2.52	0.21	11.93	17.62	2.24	7.87	4.50	1.18	3.82	2.57	0.82	3.12	5.35	1.19	4.50	2.53
G	1.54	0.47	3.28	20.39	1.45	14.02	4.47	0.74	6.04	3.11	0.51	6.14	5.96	0.91	6.52	20.32
H	4.06	0.23	17.43	23.95	4.02	5.96	4.70	1.04	4.50	4.27	0.45	9.56	7.03	0.57	12.26	16.53

gfp_{uv} in cells, selective permeation by FTS was proportional to the release of 10–40% gfp_{uv} .

The 1st and 2nd cycles removed about four times as much BSA in relation to the freezing at -75°C, decreasing four-fold the specific contents (Table 3). The concentration of 44.49 $\mu g\ gfp_{uv}/mL$ extracted by TPP from the cells, after the FTS cycles, represented 15.6% of the total gfp_{uv} ($241.19 + 44.49 = 285.68\ \mu g/mL$) released by the association of FTS followed by TPP, for culture A, resulting in specific mass (40.32 $\mu g\ gfp_{uv}/mg\ BSA$) two times higher than the equivalents obtained in the 1st and 2nd cycles. The combined procedures released four times less BSA (1.10 mg/mL), similar to that one removed from the cells directly subjected to TPP method. To perform an effective permeation, slow freezing at -75°C previously to the TPP method applied to the pelleted cells was effective for high specific gfp_{uv} mass removal.

The repetitive 1st, 2nd and 3rd FTS cycles promoted removal of gfp_{uv} . However, they created a simultaneous increase of BSA concentration, which affected the specific gfp_{uv} content, in agreement with data already obtained [10].

For cultures (B, C, D, E & F) expressing amounts lower than 100 $\mu g\ gfp_{uv}/mL$, the application of TPP method to the pelleted cells exhibited two distinct behaviors as follows:

(i) For amounts greater than 20 $\mu g\ gfp_{uv}/mL$, the TPP method was the best method, both simple and fast when applied directly to the intact cells (Table 1), resulting in concentrations of gfp_{uv}/mL and specific mass, for the cultures, respectively of (B, pool) 45.66 $\mu g\ gfp_{uv}/mL$ and 18.87 $\mu g/mg$; (C) 39.60 $\mu g\ gfp_{uv}/mL$ and 35.65 $\mu g/mg$; (D) 15.92 $\mu g\ gfp_{uv}/mL$ and 38.86 $\mu g/mg$; (E) 28.12 $\mu g\ gfp_{uv}/mL$ and 38.70 $\mu g/mg$. Comparatively, the application of the TPP method to concentrations previously permeated by FTS cycles (Table 2) resulted in total (sum of every step) equivalent concentrations for the same cultures (B: 41.17, C: 29.54, D: 18.77, E: 30.75 $\mu g/mL$). The pool (mixture) of equal parts of the successive FTS samples resulted in a ratio of about 1 gfp_{uv} : 1 BSA: 1 SM (specific mass) showing good performance. Both procedures were shown equivalent.

(ii) For amounts lower than 20 $\mu g\ gfp_{uv}/mL$ (Table 2), the concentrations attained by the direct TPP extraction from the intact cells (F: 11.56–15.78 $\mu g\ gfp_{uv}/mL$; G: 19.42 $\mu g\ gfp_{uv}/mL$; H: 9.16–16.69 $\mu g\ gfp_{uv}/mL$) were similar to those permeated (Table 3) by the 1st FTS cycle applied to cells of the same culture (F: 17.62 $\mu g\ gfp_{uv}/mL$; G: 20.39 $\mu g\ gfp_{uv}/mL$; H: 23.95 $\mu g\ gfp_{uv}/mL$) cells. Therefore, the TPP method applied to the cells subjected previously to the FTS cycles (Table 3) showed greater efficiency upon the gfp_{uv}

extraction (F: 2.53 $\mu g\ gfp_{uv}/mL$; G: 20.32 $\mu g\ gfp_{uv}/mL$; H: 16.53 $\mu g\ gfp_{uv}/mL$) corresponding to large specific contents (F: 4.50; G: 15.89; H: 16.79 $\mu g\ gfp_{uv}/mg\ BSA$). For cultures F, G, H, the maximum gfp_{uv} concentrations [F: 29.74 (=2.52+17.62+4.50+2.57+2.53); G: 49.83; H: 53.51 $\mu g\ gfp_{uv}/mL$] obtained from the combined procedures (FTS plus TPP) were 2–4 times higher than those obtained from the direct application of the TPP method to the pelleted cultures.

The percentage related to the total (-75°C+1st+2nd+3rd+TPP) concentrations of gfp_{uv} released from respective cultures (A: 203.87; B: 41.17; C: 29.54; D: 18.77; E: 30.75; F: 29.74; G: 49.83; H: 53.51) through combined FTS with TPP procedures was as follows:

(i) freezing (-75°C): A: 22%; B: 16.54%; C: 18.25%; D: 28.72%; E: 23.09%; F: 8.47%; G: 3.10%; H: 7.60%;

(ii) 1st FTS cycle: A: 28.37%; B: 18.82%; C: 46.48%; D: 37.40%; E: 53.79%; F: 59.25%; G: 40.92%; H: 44.76%;

(iii) 2nd FTS cycle: 27.07%; B: 11.97%; C: 9.38%; D: 15.02%; E: 12.07%; F: 15.13%; G: 8.97%; H: 8.78%;

(iv) 3rd FTS cycle: A: 10.59%; B: 8.00%; C: 11.41%; D: 7.35%; E: 4.10%; F: 8.64%; G: 6.24%; H: 8.00%;

(v) TPP extraction: A: 11.76%; B: 44.69%; C: 14.49%; D: 11.51%; E: 7.00%; F: 8.51%; G: 40.78%; H: 30.89%.

For cultures F, G and H, preliminary freezing at -75°C permeated the lowest amounts, about 3–8% gfp_{uv} (Table 3); however the 1st FTS cycle caused greatest removal – about 40% of the protein, promoting the most efficient permeation. Final TPP extraction applied to the cultures was important in the residual removal of the required protein up to 40% of total gfp_{uv} obtained for cultures B, G, H.

(iv) Three-partitioning phase (TPP) technique combined with sonication (Table 4)

To cultures over-expressing high concentrations of gfp_{uv} , the TPP method was applied 2–3 times directly to the pellets, while the white middle layer and the lower aqueous phase still containing gfp_{uv} , were visually detected by fluorescence with a UV lamp ($\lambda = 395\ nm$).

Sonication of the pelleted cultures before the TPP application was employed to increase the release of gfp_{uv} from the cells. Otherwise, sonication turned the homogenate unclear and hindered the TPP efficacy on fractioning proteins. The interaction of high weight proteins entrapped gfp_{uv} to the white intermediate phase, which was the layer not soluble into extraction buffer, led to the loss of the protein. Therefore, the content of BSA was increased and

Table 4: Permeation of the pellets by freezing at -75°C. Three Phase Partitioning (TPP) method applied to the pelleted culture "I" (samples 1, 2, 3, 4), culture "J" (samples 5, 6, 7, 8, 9, 10), directly (TPP) or following sonication of the pellets (S+TPP).

Pellet	Cycle	Final	BSA	<i>gfp_{uv}</i>	<i>gfp_{uv}</i>	Total	Specific mass
Sample		BSA (mg/mL)	yield (%)	(μg/mL)	yield (%)	<i>gfp_{uv}</i> (μg/mL)	<i>gfp_{uv}</i> /BSA (μg/mg)
1	(-75°C)	31.49	62.05	337.91	57.50		10.73
	1st S+TPP	5.34	10.53	137.52	23.40		25.74
	2nd S+TPP	13.92	27.42	112.28	19.11	587.71	8.07
2	(-75°C)	45.35	68.04	358.35	58.12		7.90
	1st S+TPP	5.82	87.30	165.33	26.82		28.39
	2nd S+TPP	15.47	23.21	92.854	15.06	616.53	6.00
3	(-75°C)	10.87	28.78	91.75	23.41		8.42
	1st TPP	4.01	10.65	194.30	49.67		48.41
	2nd S+TPP	22.89	60.59	105.34	26.93	391.20	4.60
4	(-75°C)	8.71	46.03	73.207	26.85		8.40
	1st TPP	2.96	15.65	134.53	49.34		45.39
	2nd S+TPP	7.25	38.30	64.94	23.82	272.68	8.96
5	(-75°C)	75.14	66.45	1633.78	46.89		21.74
	1st S+TPP	12.12	10.72	645.28	18.52		53.24
	2nd S+TPP	14.82	13.10	929.12	26.67		62.69
	3rd S+TPP	10.99	9.72	275.78	7.92	3483.96	25.10
6	(-75°C)	35.54	49.78	1262.9	37.08		35.53
	1st S+TPP	11.74	16.44	998.66	29.32		85.07
	2nd S+TPP	13.99	19.56	873.14	25.64		62.41
	3rd S+TPP	10.11	14.16	271.34	7.97	3406.04	26.84
7	(-75°C)	52.66	60.55	1606.06	37.88		30.50
	1st S+TPP	16.45	18.91	1528.08	36.04		92.90
	2nd S+TPP	13.25	15.23	875.68	20.65		66.09
	3rd S+TPP	4.60	5.29	230.03	5.43	4239.85	49.97
8	(-75°C)	14.77	32.50	584.69	25.87		39.60
	1st TPP	7.76	17.08	581.31	25.72		74.90
	2nd TPP	14.94	32.88	811.9	35.92		54.35
	3rd S+TPP	7.96	17.53	282.44	12.50	1977.90	35.46
9	(-75°C)	18.41	34.54	721.81	21.39		39.21
	1st TPP	12.62	23.68	1199.2	35.54		95.03
	2nd TPP	9.22	17.29	921.12	27.30		99.95
	3rd S+TPP	13.04	24.47	531.49	15.75	2842.13	40.75
10	(-75°C)	15.20	27.80	567.94	17.88		37.36
	1st TPP	13.23	24.18	1461.90	46.03		110.47
	2nd TPP	9.14	16.70	699.6	22.02		76.53
	3rd S+TPP	17.13	31.31	446.35	14.02	2729.42	26.05

carried with the *gfp_{uv}* extraction. For culture I, sonication plus TPP combined procedures extracted similar amounts of *gfp_{uv}* other than TPP directly applied to the cells. However, specific contents from TPP extracts (45–48 μg *gfp_{uv}*/mg) were at least twice higher than those obtained from sonication, in addition to TPP (25–28 μg *gfp_{uv}*/mg). Sonication, applied to the cells after previous TPP treatment, caused an increase of extracted *gfp_{uv}* content but affected the specific contents 3–6 fold. For samples 3 and 4 (culture I), BSA at 10 to 15% was present in nearly 50% of all *gfp_{uv}* extracted by the TPP method directly applied to the pellets, and the specific mass was the highest range at-

tained from 45–48 μg *gfp_{uv}*/mg. After sonication of those pellets, the specific content decreased between 4–8 μg *gfp_{uv}*/mg, since up to 60% of BSA was shifted with *gfp_{uv}*.

For culture J, the application of sonication previously to the TPP method did not interfere with the amount of the *gfp_{uv}* obtained. Through sample 8, it was observed that the 3rd application of the TPP method directly to the pellets did not carry out the *gfp_{uv}* fractionation. After the sonication of the pellets, the extraction of 12.50% of the protein through the TPP technique was followed by a reduction of the specific content to 35.46 μg *gfp_{uv}*/mg.

Slow freezing cycle at -75°C was responsible for the gfp_{uv} permeation up to 58% for samples 1 and 2 (culture I), and the average of 37–47% for samples 5, 6 and 7 (culture I). However, simultaneously, near 62–68% of all proteins (BSA) were carried and mixed to the required protein. These samples corresponded to high concentrations of gfp_{uv} between $338\text{ }\mu\text{g }gfp_{uv}/\text{mL}$ (sample 1) and $1634\text{ }\mu\text{g }gfp_{uv}/\text{mL}$ (sample 5). For culture I, independently of the concentrations removed of gfp_{uv} and BSA, the specific mass was equivalent to 8–10 $\mu\text{g }gfp_{uv}/\text{mg}$, also for samples 3 and 4, when the freezing removal of gfp_{uv} varied from 23–26%.

For culture I, even though the concentration of gfp_{uv} ranged from $568\text{ }\mu\text{g}/\text{mL}$ (sample 10) to $1634\text{ }\mu\text{g}/\text{mL}$ (sample 5), 39% (samples 8 and 9), corresponded to half gfp_{uv} extracted – respectively, $584.69\text{ }\mu\text{g}/\text{mL}$ and $721.81\text{ }\mu\text{g}/\text{mL}$.

The uncontrolled slow freezing (-75°C) speed of the pellets caused variations in the permeation of the pelleted cells.

(v) Three-partitioning phase (TPP) technique directly applied (Table 4)

For samples 3 and 4 (culture I), TPP directly applied to previously frozen cells, extracted two times more gfp_{uv} (49.34–49.67%) than prior freezing permeation (23.41–26.85%), corresponding to a five-fold increase of the specific contents (from $8.4\text{ }\mu\text{g}/\text{mg}$ to $45.39\text{--}48.41\text{ }\mu\text{g}/\text{mg}$), which showed simultaneous ability by the TPP method of extraction, purity and concentration of gfp_{uv} in the aqueous phase. The sonication following procedure displaced 6 times more BSA mixed to gfp_{uv} reducing up to 10 times the specific contents (sample 3), affecting the purity of the homogenate.

The TPP method applied directly to samples 8, 9, and 10 (culture I) attained the best specific content ranging from $74.90\text{ }\mu\text{g}/\text{mg}$ to $110.47\text{ }\mu\text{g}/\text{mg}$ for the first application and from $54.35\text{ }\mu\text{g}/\text{mg}$ to $76.53\text{ }\mu\text{g}/\text{mg}$ for the second turn. Sonication following TPP application to the extracted pellets caused a three-fold decrease of specific contents ($26.05\text{ }\mu\text{g}/\text{mg}$, sample 10), confirming that sonication had a negative effect on the isolation and purification procedures of gfp_{uv} .

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Anexo 06

“Influence of Culture Conditions on the Expression of Green Fluorescent Protein (*GFPuv*) by *Escherichia coli*”.

Trabalho apresentado na forma de poster no
25th *Symposium on Biotechnology for Fuels and Chemicals*,
Colorado, Maio de 2003

Artigo submetido à publicação em: *Biotechnology Progress*, 2003

Objetivo

Este trabalho visou a otimização das condições de crescimento de *E. coli* DH5- α e, conseqüentemente, a otimização da produção de *GFPuv*.

As variáveis estudadas na influência do crescimento bacteriano, conseqüentemente na expressão de *GFPuv* foram: (i) o inóculo inicial; (ii) o armazenamento à temperatura de 4°C antes da incubação; (iii) velocidade de agitação; (iv) a adição de IPTG em diferentes densidades celulares e, (v) o tempo total de incubação.

Conclusões

As condições de cultivo (37°C/24 h de incubação) as quais forneceram maior produção de *GFPuv* foram: (i) armazenamento a 4°C por 24 h; (ii) velocidade de agitação de 100 rpm; (iii) concentração de IPTG entre 0.05 mM e 0.5 mM; e, (iv) adição de IPTG a OD_{660nm} = 0,01, correspondendo a densidade celular no início da fase lag de crescimento. O armazenamento, a velocidade de agitação e a densidade celular em que o IPTG foi adicionado mostraram grande influência na expressão de *GFPuv*. A adição de IPTG em diferentes estágios do crescimento bacteriano não interferiu na taxa exponencial constante.

Influence of Culture Conditions on the Expression of Green Fluorescent Protein (*GFPuv*) by *Escherichia coli*.

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ABSTRACT

The recombinant green fluorescent protein (*GFPuv*) was expressed by transformed cells of *Escherichia coli* DH5- α , grown in LB/amp broth at 37°C. The seeded broth was stored at 4°C prior to further incubation on a rotary shaker for 8 h and 24 h. To evaluate the effectiveness of different parameters to improve the expression of *GFPuv* by *E. coli*, nine cultures with four variable culturing conditions were set up for eight assays by a fractional factorial (2^{4-1}) design at two levels: (i) the effect of storing the starting culture in LB/amp broth at 4°C for 24 h and 48 h (“storage”) prior to incubation at 37°C; (ii) the effect of agitation speed (100, 150 and 200 rpm); (iii) the concentration of IPTG (isopropyl- β -D-thiogalactopyranoside, final concentrations of 0.05, 0.275 and 0.5 mM); (iv) the addition of IPTG at set cell densities (OD_{660} between 0.01 and 0.8). The expressed *GFPuv* was extracted by the three phase partitioning method (TPP) and further purified with a methyl HIC column. The fluorescence intensity of the samples were related to $\mu\text{g GFPuv/mL}$. The culture conditions (37°C/24 h incubation) which provided the highest yields of *GFPuv* were: (i) storage at 4°C for 24 h; (ii) agitation speed at 100 rpm; (iii) the concentration of IPTG at 0.5 mM; (iv) the addition of IPTG at cell densities at $OD_{660} = 0.01$. Agitation speed and IPTG concentration showed, respectively, greater negative and positive influences on *GFPuv* expression at the beginning of the logarithmic phase.

Key words: *Green fluorescent protein (GFPuv), Escherichia coli* DH5- α , *growth conditions, three- phase- partitioning extraction (TPP)*.

INTRODUCTION

Bioluminescence is a natural phenomenon that has been observed in many terrestrial and marine organisms. The green fluorescent protein (*GFP*) is a 27-29 kDa protein that provides a unique environment for three residues in its primary sequence to act as a fluorophore (1). Recombinant *GFPuv* was developed by introducing point mutations that replaced three amino acid codons in the native *GFP* DNA sequence (2,3). *GFPuv* is a simple and versatile tool for in vitro and in vivo work. It has been used extensively in studies of genetic expression in that it can be successfully expressed by live cells (1,2,3,4) and easily seen under a UV light (4). *GFPuv* is intrinsically expressed fluorescent and requires no exogenous factors other than the appropriate excitation wavelengths. *GFPuv* is expressed two to three times faster in *E. coli* and exhibits eighteen-fold greater fluorescence intensity than native *GFP*. Its expression depends on the amount of inductor and the timing of induction. With the excitation maxima at 394-395 nm and 508-509 nm for emission (1,2), the fluorescence characteristics of *GFPuv* can be quantified in situ using a variety of techniques using fluorescence microscopy, flow cytometry and spectrofluorometry.

Shi and Su (5) studied the expression of green fluorescent protein on the external surface of *E. coli* cells and demonstrated that surface localized *GFPuv* can be used as a quantitative reporter to study gene expression.

Geoffroy *et al.* (6) showed that intracellular *GFP* in lactic acid bacteria, which were developed as live vaccine carriers, can be a useful marker to monitor the bacteria localization when administered to animal hosts or to analyze their interactions with different cell types.

Webb *et al.* (7) investigated the use of *GFP* as a potential reporter molecule to monitor fungal susceptibility to antimicrobial compounds. The authors verified the relationship between fluorescence levels in *Aureobasidium pullulans* suspensions and the number of viable cells (CFU plate counts). Further, losses of *GFP* fluorescence from *A. pullulans* cells was correlated with a decrease in the number of viable cells.

OBJECTIVES

The importance of extracting purified *GFPuv* from *E. coli* growth has been the object of other research (8, 9, 10) but not with the emphasis that was investigated in the present work. The purpose of this study was to investigate the effect of various culture conditions on the expression of *GFPuv* in transformed *E. coli*. To improve the expression of *GFPuv*, transformed *E. coli* DH5- α cells were subjected to various conditions to study the effects of: (i) storing the starting culture at 4°C (“storage”) prior to incubation at 37°C, (ii) agitation speed; (iii) the addition of IPTG at set cell densities (OD₆₆₀); and (iv) the concentration of IPTG added to the culture.

MATERIALS AND METHODS

Transformation. *Escherichia coli* DH5- α were transformed with *pGFPuv* (Clontech, CA, USA), by the standard calcium chloride method (11), following the procedure described by Vessoni Penna *et al.* (8). The recombinant GFPuv is under tight control of the *lacZ* protein β -galactosidase promoter/repressor that allows induction and high expression using isopropyl- β -D-thiogalactopyranoside (IPTG, USB/Italy) (1,2,3). The vector includes the *bla* gene for ampicillin (amp, Boehringer, Mannheim, Germany) selection. The transformed cells were stored at -75 °C into Luria Bertani broth (LB: Difco, USB/Mexico) supplemented with 100 μ g/mL ampicillin and 50% glycerol.

Experimental Design. To evaluate the effectiveness of different parameters to improve the expression of *GFPuv* by *E. coli*, nine culture conditions were set up by a fractional factorial (2^{4-1}) design at two levels: (i) the effect of storing the starting culture in LB/amp broth at 4°C for 24 h, 36 h and 48 h (“storage”) prior to incubation at 37°C; (ii) the effect of rotary speed (100, 150 and 200 rpm); (iii) the effect of the addition of IPTG at set cell densities (OD₆₆₀ between 0.01 and 0.8) corresponding to cultures at 10^3 - 10^7 CFU/mL; (iv) the concentration of IPTG with final concentrations of 0.05, 0.275 and 0.5 mM; (v) incubation of the culture at 37°C a period of 8 h or 24 h (Table 01).

Inoculum. A 24 h culture of transformed *E. coli* (LB/amp broth; 37°C/100 rpm) was transferred on to the surface of LB/amp/IPTG agar and incubated (37°C/24h). Illuminating the agar surface with a hand-held long UV lamp ($\lambda = 360$ to 395 nm; Model UVL 4, UVP, CA, USA), isolated green fluorescent colonies were picked and transferred to 25 mL LB/amp broth in 250 mL Erlenmeyer flasks (“starter cultures”). The starter cultures were incubated (37°C/100 rpm) until the cell density of 0.0054 to 0.026 absorbance units ($10^4 - 10^5$ CFU/mL) were obtained as measured by OD₆₆₀ with a spectrophotometer (Beckman DU-600, CA, USA). The inoculum of 1.0 mL was transferred to each of 32 erlenmeyers (250 mL) containing 25 mL of LB/amp. The seeded 25 mL LB/amp broth in erlenmeyers were stored at 4°C previously being shaken incubated (Tecnal model TE 240, SP, Br) at 37°C.

Induction by IPTG addition. The expression cultures were incubated (37°C) until the broth cultures attained a set OD₆₆₀ for the addition of IPTG (Table 1). Expression cultures from corresponding flasks (two flasks/hour) were assayed every hour for cell density: (i) OD₆₆₀ with LB/amp broth in the reference cell; (ii) dried biomass related to *GFPuv* expression ($\mu\text{g GFPuv} / \text{mg DCW}$; DCW=dried cellular weight) from cells retained on the surface of a 0.22 μm membrane (Millipore, SP, Brazil) and submitted to 105°C around 24h, attaining constant weight; After IPTG addition, the *GFPuv* expression by induced cells was accompanied every hour by UV light at $\lambda = 395 \text{ nm}$.

Extraction of GFPuv by three- phase- partitioning extraction (TPP) method (12,9) and purification (10). The cultures were centrifuged (1000 g/ 30 min/ 4 °C). The pellets were observed under UV light (395 nm), resuspended into cold extraction buffer solution (XE: 25 mM Tris-HCl, pH 8.0 - Trizma® Base, Sigma, MO, USA; 1.0 mM β -mercaptoethanol " β -ME", Pharmacia Biotech, Sweden; 0.1 mM phenylmethylsulphonylfluoride "PMSF", USB, Switzerland) and subjected to direct extraction by TPP method. To each 450 μL of cell suspension, 300 μL of 4 M $(\text{NH}_4)_2\text{SO}_4$ (1.6 M final concentration) and 750 μL of *t*-butanol (ratio 1:1) were added. The mixtures were vortexed for 2.0 min, centrifuged at 6,000 g/ 10 min. The three phases formed were collected. After the *t*-butanol upper phase and the white interfacial precipitate were removed, another equal volume of *t*-butanol was mixed with the lower aqueous phase and centrifuged. The upper phase of the system was discarded. The interfacial green phase was collected and dissolved in 1000 mL XE buffer. Purification of the extracts was made through methyl support hydrophobic interaction chromatography (HIC), and the *GFPuv* 1mL-portion was eluted in buffer solution (10 mM Tris-HCl, 10 mM EDTA), and stored at 4 °C.

The fluorescence intensity of GFPuv was measured in the eluted samples in a Fluorescence Spectrophotometer (RF – 5301 PC – Shimadzu Corporation, Kyoto, Japan), with an excitation filter of 394 nm and an emission filter of 505 nm. The fluorescence intensity of the samples was compared to a standard curve (1) ($\text{GFPuv } \mu\text{g/mL} = [(\text{fluorescence intensity}) + 33.078]/38.943$; $R^2 = 0.997$) and the concentration was expressed in $\mu\text{g GFPuv} / \text{mL}$. The standard curve was prepared using known amounts (between 1.32 $\mu\text{g/mL}$ and 10.00 $\mu\text{g/mL}$) of standard

recombinant *GFPuv* (Clontech) diluted in the same buffer solution (10 mM Tris-HCl, 10 mM EDTA, pH 8.0).

Statistical Growth Variables. The effects of the variables, interaction coefficients (95%), correlation matrix for estimated parameters, respective confidence intervals, significance levels ($p < 0.05$) and regression variance analysis (ANOVA) (13) were calculated through SGWIN program (Statgraphics Plus for Windows version 3 - Statistical Graphics Corporation, Rockville, MD, 1997). The four variables (x) considered in regression analysis were taken by dimensionless values over the same (-1) to (+1) range. The maximum (+1), the intermediate (0) and minimum (-1) codified ranges for each independent variable, shown in Table 1, were: x_1 = storage at 4°C (24 h, 36 h, 48 h) x_2 = agitation speed (100 rpm, 150 rpm and 200 rpm); x_3 = the effect of the addition of IPTG at set OD₆₆₀ (0.01, 0.40 and 0.80); x_4 = the final concentrations of IPTG (0.05 mM, 0.275 mM and 0.5 mM); x_5 = the incubation time at 37°C for 8 h ($x_5 = -1$) and 24 h ($x_5 = +1$). The intermediate levels in code units were given by the equations: codified variable (x_n) = $\{[(\text{effect studied}) - (\text{maximum level} + \text{minimum level})/2] / [(\text{maximum level} - \text{minimum level})/2]\}$.

RESULTS

E. coli Growth Rates and *GFPuv* Expression.

Escherichia coli was the species chosen to produce *GFPuv*, because the bacterium has a well-defined genetic map. The strain, *E. coli* DH-5 α , achieved competence easily and has been shown to be transformed efficiently with p*GFPuv*, a circular DNA plasmid constructed from the pUC that provides for a high replication number and offers a suitable vector for the production of large quantities of p*GFPuv* (2). The expression of *GFPuv* is under tight control of the *lacZ* protein β -galactosidase promoter/repressor (1) and it was continuously induced with a concentration varying from 0.05 mM to 0.5 mM (w/v) IPTG added to the *E. coli* culture in different growth phases, as measured by the cell density (OD₆₆₀).

During the exponential phase, *E. coli* divides at a constant rate and the increase of the number of cells was shown to be directly proportional to *GFPuv* expression after induction with IPTG at set culture OD₆₆₀ (0.01, 0.40 and 0.80)

conditions (Figure 1). Considering incubation at 37°C for 8 h ($x_5 = -1$) or 24 h ($x_5 = +1$), no lag phase was exhibited. Independent on the growth phase for the IPTG addition, at the beginning (OD = 0.01) or at the end (OD = 0.8) of the logarithmic phase, the remaining stationary phase was long enough for the over expression of *GFPuv* by the cells of *E. coli* (Figure 1). On the other hand, for 8 h ($x_5 = -1$) incubation, the expression of *GFPuv* by the *E.coli* cells was dependent on the addition of IPTG at the beginning of the log phase (Table 2, Figure 2).

Considering 24 h ($x_5 = +1$) of incubation and that the expression of *GFPuv* is directly proportional to the cell growth, the increase exponential for *GFPuv* expression rate was $\mu_{GFPuv} = 0.203 \text{ h}^{-1}$, approximately 16 times less than the exponential growth rate ($\mu = 1.284 \text{ h}^{-1}$) for the *E. coli* cells (14). The constant exponential rate for culture growth varied from 0.99 h^{-1} to 2.10 h^{-1} .

Considering 8 h ($x_5 = -1$) of incubation and that the IPTG was added to the cultures: (i) directly incubated at 37°C/100rpm at OD₆₆₀ between 0.77 and 0.84, at the end of logarithmic phase; and (ii) previously stored refrigerated (4°C/ 24h) before incubation (37°C/100rpm), the IPTG was added at OD₆₆₀ between 0.023 and 0.044 at the beginning of logarithmic phase, VP *et al.* (10, 13) verified that the average exponential rate constant ranged from (i) 1.36 h^{-1} (culture directly incubated) to 2.10 h^{-1} (stored culture), between 1.96 and 3.03 generations/h. The refrigeration of cultures before incubation positively influenced the cell growth and the addition of IPTG in different stages of growth process did not interfere in the exponential rate constant.

Optimal Conditions for the Expression of GFPuv during 8 h ($x_5 = -1$) and 24 h ($x_5 = +1$) Incubation Times for E. coli Cultures.

The influence of the set culture conditions in the expression of *GFPuv* (Table 2) was studied under nine groups, the storage (4°C) of seeded broth before incubation at 37°C, the agitation speed of the culture for 8 h ($x_5 = -1$) and 24 h ($x_5 = +1$) incubation at 37°C, and the addition of IPTG to the culture at different concentrations and cell densities, respectively, as shown in Table 1.

The variables were analyzed by applying a multiple linear regression to the data related to the concentrations of expressed *GFPuv* obtained from the induced *E. coli* cultures. Using the TPP extraction method followed by purification in methyl HIC columns, proteins (other than *GFPuv*), nucleic acids and other molecules were separated from *GFPuv* to provide a recovery of more than 90% of the total *GFPuv* expressed.

The ANOVA analysis showed the results by fitting a multiple linear regression model to describe the relationship between $\mu\text{g } GFPuv / \text{mL}$ and the independent variables. The attained quadratic polynomial model is: $\mu\text{g } GFPuv / \text{mL} = 10.28 - 1.13 x_1 - 5.65 x_3 + 8.28 x_5 - 1.76 x_1 x_5 - 5.38 x_2 x_5 + 1.21 x_4 x_5$ (main equation, shown on Tables 3 and 4), for the independent variables: x_1 = period (h) of storage at 4°C; x_2 = rotary speed (rpm); x_3 = cell densities (OD_{660}) upon the addition of IPTG; x_4 = concentration of IPTG (mM) added, and x_5 = culture incubation at 37°C, set at 8 h and 24 h. Since the p-value in the ANOVA is less than 0.01, there is a statistically significant relationship between the variables at the 99% confidence levels. Therefore, the analysis of the statistical coefficients (Appendix 1), at the significance level of $p < 0.01$, provided statistical significance to the polynomial models and consistency to the estimated regression coefficients. Therefore, its geometric interpretations, such as surface response graphs (Figure 2) for the first-order equations are reliable, showing the effects exhibited by the independent variables.

The greatest improvement in the GFPuv expression from cultures for extreme culture conditions:

For 8 h ($x_5 = -1$) of incubation

With 8 h ($x_5 = -1$) culture incubation at 37°C, the extracted concentration of *GFPuv* in the fitted equations (Table 3) is proportional to the positive coefficient related to rotary speed (x_2) and to the negative coefficient of the culture cell density (OD_{660} , x_4) at which IPTG is added to the culture of *E. coli*. This observation is confirmed by the 3rd case when storage is kept at 4°C for maximum 48 h ($x_1 = +1$) and the final IPTG concentration added at minimum concentration of 0.05 mM ($x_4 = -1$). By the analyses of the extreme conditions: (i) the highest concentration of *GFPuv* extracted (14.87 µg *GFPuv*/mL) is related to maximum 200 rpm ($x_2 = +1$) agitation speed and minimum cell densities ($OD_{660} = 0.01$, $x_3 = -1$) at the addition of IPTG; (ii) the worst condition that provided no expression of *GFPuv* (-7.19 µg *GFPuv*/mL) was evident with the minimum 100 rpm ($x_2 = -1$) rotary speed for an addition of IPTG at the highest OD_{660} of 0.8 ($x_3 = +1$); (iii) equivalent effect on the expression of around 4 µg *GFPuv*/mL is verified for both x_2 and x_3 at minimum levels (100 rpm and 0.01 OD_{660}) or at maximum levels (200 rpm and 0.8 OD_{660}). The performance of these extreme culture conditions presented by the 3rd case (Table 3) upon *GFPuv* is illustrated in the Figure 2, (i).

For 24 h ($x_5 = +1$) of incubation at 37°C

For 24 h ($x_5 = +1$) incubation at 37°C, setting the storage at minimum 24 h ($x_1 = -1$) at 4°C and IPTG maximum concentration of 0.5 mM ($x_4 = +1$), we observe through the fitted equations of the 4th case (Table 4), that the influence of both negative coefficients for rotary speed (x_2) and cell OD_{660} density (x_3) on the expression of *GFPuv*, attained the highest concentration of 33.68 µg *GFPuv*/mL by keeping their minimum levels of rotary speed (100 rpm, $x_2 = -1$) and OD_{660} cell density (0.01 OD_{660} , $x_3 = -1$). Otherwise, at maximum levels for both variables, 200 rpm ($x_2 = +1$) rotary speed and 0.8 OD_{660} ($x_3 = +1$) cell densities for the addition of IPTG to the culture, the expression of *GFPuv* by *E. coli* decreases three times to

11.62 $\mu\text{g GFPuv/mL}$. The performance of these extreme culture conditions presented by the 4th case (Table 4) upon GFPuv is illustrated in the Figure 2, (ii).

Comparing the highest expression of GFPuv by *E.coli* after incubations at 8 h ($x_5 = -1$) and 24 h ($x_5 = +1$) at 37°C, we observed that although the GFPuv expression begins following the addition of IPTG (maximum and minimum concentrations), the rotary speed was shown to exhibit a remarkable influence on the increase in cell density at the minimum level of 0.01 OD₆₆₀ at the beginning of the logarithmic phase, when the newly divided cells are induced by IPTG to express GFPuv. Maximum rotary speed of 200 rpm ($x_2 = +1$) for a shorter incubation, 8 h ($x_5 = -1$), almost compensated, without success, the minimum speed at 100 rpm ($x_2 = -1$) for a longer 24 h incubation ($x_5 = +1$) at 37°C, unless the 8 h ($x_5 = -1$) incubation provided half the concentration of GFPuv obtained for 24 h incubation.

Another remarkable positive effect on GFPuv expression was the storage at 4°C for 24 h ($x_1 = -1$) and 48 h ($x_1 = +1$) of the starter culture and then 8 h ($x_5 = -1$) and 24 h ($x_5 = +1$) incubation at 37°C. The expression of the highest concentrations of GFPuv was shown to be independent of the time in storage. The storage extends the lag phase without any detrimental effect to cell growth. On the contrary, it seems to increase the ability of the cells to multiply when incubated at optimum growth temperature (37°C), at high speed (200 rpm, $x_2 = +1$) for a shorter incubation, 8 h ($x_5 = -1$), or at low speed (100 rpm, $x_2 = -1$) for a longer incubation, 24 h ($x_5 = +1$).

Equivalent conditions to better achieved expressions of GFPuv.

(i) For 8 h ($x_5 = -1$), incubation at 37°C

With 8 h ($x_5 = -1$) incubation at 37°C (Table 3), the influence of the storage (x_1) can be noted in the 2nd case over the expression attained of 13.61 $\mu\text{g GFPuv/mL}$, which concentration was very close to the highest concentration of 14.87 $\mu\text{g GFPuv/mL}$ obtained for the same conditions (200 rpm and 0.01 OD₆₆₀) with storage set at maximum level ($x_1 = +1$) of 48 h at 4°C, the 3rd case .

(ii) For 24 h ($x_5 = +1$) incubation at 37°C:

The final minimum (0.05 mM, $x_4 = +1$) and maximum (0.5 mM, $x_4 = -1$) IPTG concentrations, added at the minimum OD₆₆₀ cell density ($x_3 = -1$, OD₆₆₀ = 0.01) with the culture kept at 100 rpm ($x_2 = -1$), the minimum rotary speed, seems not to interfere over the expression of 31.26 µgGFPuv/mL (2th case) and 33.68 µgGFPuv/mL (4th case), respectively, with both cultures incubated for 24 h ($x_5 = +1$) at 37°C (Table 4).

Intermediate, Lower and Lowest expressions of GFPuv by cultures of transformed cells of E. coli

For 8 h ($x_5 = -1$), incubation at 37°C

With 8 h ($x_5 = -1$) incubation at 37°C (Table 3), the lowest expression of 0.42 µg GFPuv/mL was attained at 24 h ($x_1 = -1$) storage at 4°C and maximum IPTG concentration (0.5 mM, $x_4 = +1$) added at the start of the log phase (OD₆₆₀ = 0.01, $x_3 = -1$) for minimum 100 rpm level ($x_2 = -1$). Therefore, changing the rotary speed to the maximum level of 200 rpm ($x_2 = +1$), the expression of GFPuv increased 26 times to 11.19 µg GFPuv/mL.

For 24 h ($x_5 = +1$) incubation at 37°C

On the other hand, as shown in the 3rd case, a culture grown at maximum rotary speed, 200 rpm ($x_2 = +1$), for 24 h incubation ($x_5 = +1$, Table 4) expressed the lowest concentration of GFPuv, 3.42 µg GFPuv/mL, after maximum storage (4°C/48 h, $x_1 = +1$) of the cells and the lowest amount of IPTG added (0.05 mM; $x_4 = -1$) at maximum OD₆₆₀ of 0.8 ($x_3 = +1$) cell density, which corresponds to the end of the growth log phase, as demonstrated in Figure 2, (iii). Therefore, the GFPuv concentration (25.48 µg /mL) increases 7.5 times on slowing the culture rotary speed to 100 rpm ($x_2 = -1$) even after adding the lowest amount of IPTG at the beginning of the log phase (OD₆₆₀ = 0.01, $x_3 = -1$) of the stored culture at 4°C/48 h ($x_1 = +1$). However, this obtained expression of 25.48 µg GFPuv/mL is just 1.5 times less than the highest concentration of GFPuv for the best conditions of growth with parallel expression, the 4th case (Table 4).

For every case and 24 h ($x_5 = +1$) incubation at 37°C, intermediate expressions of *GFPuv* were obtained from the cultures developed at maximum speed and IPTG added at minimum OD₆₆₀ cell density or the cultures grown at the minimum rotary speed and IPTG added at the maximum OD₆₆₀. The intermediate estimated *GFPuv* concentrations are around (i) 17 µg *GFPuv*/mL, 1st case; (ii) 20 µg *GFPuv*/mL, 2nd case; (iii) 14 µg *GFPuv*/mL, 3rd case; (iv) 22 µg *GFPuv*/mL, 4th case; (v) 18 µg *GFPuv*/mL, 5th case. The higher intermediate *GFPuv* concentrations predicted by the 2nd and 4th cases (Table 4) correspond to minimum storage of 24 h ($x_1 = -1$) at 4°C, independent of minimum or maximum IPTG concentration, respectively.

DISCUSSION

The lag phase of microbial growth represents the time period needed for bacteria to adapt to a new environment before cell division commences. Certain relevant variables affecting lag time should be taken into account, particularly the physiological state of the inoculum and the inoculum size. Robinson *et al.* (15) investigated the effect of inoculum size on the population lag time of *Listeria monocytogenes*, under conditions of salt stress and the heterogeneity in lag times of inocula containing very low cell numbers. The specific growth rates estimated from the detection times were 1.12 and 1.22 h⁻¹, with correlates with our data between 0.99 h⁻¹ to 2.10 h⁻¹ greater than parallel expression ($\mu = 0.20$ h⁻¹) of *GFPuv* by the *E. coli* cells. In this work, the optimal cultivation condition at 37°C and 100 rpm for *E. coli* DH5- α cells was shown to be independent of inoculum storage at 4°C for 24 h or 48 h before incubation for 24 h. On the other hand, the storage at 48 h and rotary speed at 200 rpm for 8 h incubation at 37°C increased the concentration of *GFPuv* during the logarithmic phase, with induction time at low cell densities.

Shi and Su (5) examined the highest IPTG concentration and optimal induction time over the expression of green fluorescent protein on the extracellular surface of *E. coli* JM109 (pUMC101) cells cultivated in two temperatures, 25°C and 37°C. The authors verified that growth of *E. coli* at 25°C/24 h resulted in a two-fold

increase in fluorescence signal, but with a longer doubling time; cell growth at 37°C resulted in no increase in culture fluorescence relative to background cellular fluorescence. The authors also verified that the timing of induction showed a significant effect upon cell growth when the culture was induced at a lower density than $OD_{660} < 1.0$ for concentrations greater than 0.05 mM IPTG. In the present work, it was verified that the range of IPTG from 0.05 mM to 0.5 mM added to the cultures was independent on the cell density (DO_{660} between 0.01 and 0.8), however the induction timing, addition of IPTG, at the beginning of exponential phase of growth favored the expression of *GFPuv* by the *E.coli*.

Lactic acid bacteria are important for their acidifying properties, their metabolism and their ability to interfere with the growth of pathogenic bacteria. Palencia *et al.* (16) studied the expression of green fluorescent protein in *Lactococcus lactis*, following the growth of cultures by spectrophotometry and their levels of fluorescence at pH 8.0 by fluorescence spectroscopy. The results showed that the presence of *GFP* did not affect the growth of the cells. In addition, the fluorescence of strains increased linearly with the cell mass, indicating that the intrinsic fluorescence of the cells, as well as the fluorescence of *GFP*, is correlated with the cell growth.

CONCLUSIONS

The culture conditions (37°C/24 h incubation) which provided the highest yields of *GFPuv* were: (i) storage at 4°C for 24 h; (ii) rotary speed at 100 rpm; (iii) the concentration of IPTG between 0.05 mM and 0.5 mM; (iv) the addition of IPTG at $OD_{660} = 0.01$ corresponding to cell densities at the beginning of the lag growth phase. Storage, rotary speed and the cell density at which the IPTG is added, showed greater influences on *GFPuv* expression upon the *E. coli* cultures. The addition of IPTG in different stages of growth process did not interfere in the exponential rate constant.

Appendix 1:

Main equation:

$$\mu\text{g GFP}_{uv}/\text{mL} = 10.28 - 1.13 x_1 - 5.65 x_3 + 8.28 x_5 - 1.76 x_1 x_5 - 5.38 x_2 x_5 + 1.21 x_4 x_5$$

Independent variables: x_1 = period (h) of storage at 4°C; x_2 = rotary speed (rpm); x_3 = cell densities (OD₆₆₀) upon the addition of IPTG; x_4 = concentration of IPTG (mM) added, and x_5 = culture incubation at 37°C, set at either 8 h or 24 h. *Statistical coefficients:*

$p < 0.01$ significance level

$n = 170$ observations

$p = 7$ coefficients

$R^2 = 0.943$ multiple determination for $(n-1) = 169$ degrees of freedom (d.f.)

$R^2 = 0.941$ (d.f. = $p - 1 = 6$)

Mean absolute error = 1.92467 (average value of the residuals)

SE = standard error = 2.35 ($n - 1 = 169$ d.f.) deviation of the residuals from the estimate.

Model Student's $t_{(0.01/2)} > \pm 2.45$

Model $F_{\text{-ratio}} (449.33) > F_{\text{critical}} (0.99; p-1=6; n-1= 169; n-p=163) = 2.80$

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Table 1: The expression of *GFPuv* by *E.coli* from nine cultures (groups) set up by a fractional factorial (2^{4-1}) design at two levels. The cultures were incubated at 37°C on a rotary shaker for 8 h ($x_5 = -1$) and for 24 h ($x_5 = +1$).

Culture Group	x_1	x_2	x_3	x_4
1	-	-	-	-
2	+	-	-	+
3	-	+	-	+
4	+	+	-	+
5	-	-	+	+
6	+	-	+	-
7	-	+	+	-
8	+	+	+	+
9	0	0	0	0

Where:

Independent Variables		(-)	0	(+)
x_1	Storage 4°C (h)	24	36	48
x_2	Agitation speed (rpm)	100	150	200
x_3	¹OD_{660nm} add IPTG	0.01	0.40	0.80
x_4	²IPTG (mM)	0.050	0.275	0.500

¹The addition of IPTG at different cell densities

² The final concentration of IPTG added

Table 2. Extracted concentrations of *GFPuv* ($\mu\text{g GFPuv}/\text{mL}$) and effects of expression ($\mu\text{g GFPuv}/\text{mg DCW}$) derived from *E.coli* cells cultivated at set conditions for standard periods of 8 h ($x_5 = -1$) and 24 h ($x_5 = +1$) incubation at 37°C .

Variables for standard 8 h incubation ($x_5 = -1$)							
¹ Assay	² Group	Storage	RPM	IPTG (mM)	OD ₆₆₀ IPTG	Incubation (h)	DCW mg/mL
		x_1	x_2	x_3	x_4	x_6	
1	1	-1	-1	-1	-0.99	-1	0.24
2	2	1	-1	1	-0.98	-1	0.43
3	2	1	-1	1	-0.98	-1	0.43
4	2	1	-1	1	-0.98	-1	0.47
5	3	1	1	-1	-0.98	-1	1.76
6	7	-1	1	-1	0.99	-1	1.68
7	7	-1	1	-1	0.99	-1	1.68
Concentration							Specific productivity $\mu\text{g GFPuv}/\text{mg DCW}$
$\mu\text{g GFPuv} / \text{mL}$							mean $\pm$ CI
							mean $\pm$ CI
							4.85 $\pm$ 0.47
							3.18 $\pm$ 0.51
							3.91 $\pm$ 0.52
							2.44 $\pm$ 0.16
							9.20 $\pm$ 1.54
							2.36 $\pm$ 0.34
							2.36 $\pm$ 0.34

Variables for standard 24 h culture incubation ($x_5 = +1$)							
¹ Assay	² Group	Storage	RPM	IPTG (mM)	OD ₆₆₀ IPTG	Incubation (h)	DCW mg/mL
		x_1	x_2	x_3	x_4	x_6	
1	1	-1	-1	-1	-0.98	1	1.98
2	1	-1	-1	-1	-0.99	1	2.03
3	2	1	-1	1	-1.00	1	2.14
4	2	1	-1	1	-0.99	1	2.19
5	3	1	1	-1	-0.99	1	2.06
6	3	1	1	-1	-0.99	1	2.12
7	4	-1	1	1	-0.99	1	2.16
8	4	-1	1	1	-0.98	1	1.95
Concentration							Specific productivity $\mu\text{g GFPuv}/\text{mg DCW}$
$\mu\text{g GFPuv} / \text{mL}$							mean $\pm$ CI
							mean $\pm$ CI
							16.97 $\pm$ 0.54
							15.24 $\pm$ 0.77
							12.00 $\pm$ 0.86
							13.30 $\pm$ 0.82
							7.05 $\pm$ 0.40
							7.68 $\pm$ 0.52
							10.52 $\pm$ 0.60
							10.86 $\pm$ 0.56

9	5	1	-1	-1	0.12	1	2.05	19.24±2.20	9.38±1.08
10	5	1	-1	-1	0.31	1	2.09	21.76±0.58	10.43±0.28
11	5	1	-1	-1	-0.35	1	2.14	22.20±1.38	10.28±0.65
12	5	1	-1	-1	-0.098	1	2.16	17.32±0.44	8.03±0.20
13	6	-1	-1	1	-0.56	1	1.98	31.98±2.02	16.19±1.02
14	6	-1	-1	1	-0.53	1	2.02	30.64±1.46	15.17±0.73
15	6	-1	-1	1	0.37	1	2.07	24.78±1.88	11.97±0.91
16	7	-1	1	-1	1.00	1	2.15	11.47±0.82	5.33±0.38
17	7	-1	1	-1	0.69	1	2.09	9.84±2.74	4.71±1.31
18	7	-1	1	-1	0.73	1	2.15	7.26±0.47	3.38±0.22
19	8	1	1	1	0.75	1	2.22	9.57±0.51	4.30±0.23
20	8	1	1	1	0.026	1	2.10	9.67±0.33	4.61±0.23
21	9	0	0	0	0.49	1	2.41	12.76±0.91	5.29±0.38
22	9	0	0	0	0.13	1	2.19	20.16±1.45	9.19±0.66
23	9	0	0	0	0.013	1	2.31	18.71±1.00	8.11±0.43

¹Assay= the order in which the experiments were carried out.

²Group= the set conditions for every experiment carried out.

³CI = Confidence Interval, $p < 0.05$; $n = 10$ observations

Table 3. Fitted models for the expression of GFPuv ($\mu\text{g GFPuv/ mL}$) by setting up levels for the factors: x_1 = storage at 4°C; x_2 = rotary speed (RPM); x_3 = addition of IPTG at OD₆₀₀ (OD₆₀₀ IPTG); x_4 = final concentration of IPTG (mM); with x_5 = -1, incubation set at 37°C for 8 h.

$\mu\text{g GFP}_{uv}/\text{mL} = 10.28 - 1.13\text{ }x_1 - 5.65\text{ }x_3 + 8.28\text{ }x_5 - 1.76\text{ }x_1x_5 - 5.38\text{ }x_2x_5 + 1.21\text{ }x_4x_5$				
FACTOR RELATION	FITTED EQUATION ($\mu\text{g GFP}_{uv}/\text{mL}$)	Extreme Conditions	$\mu\text{g GFP}_{uv}/\text{mL}$	ASSAYS
1 st Case: $x_1 = +1$; $x_4 = +1$; $x_5 = -1$				
$\mu\text{g GFP}_{uv}/\text{mL} = 1.41 + 5.38\text{ }x_2 - 5.65\text{ }x_3$				
		$x_2 = +1$ $x_3 = +1$	1.15	3
		$x_2 = -1$ $x_3 = -1$	1.68	
		$x_2 = -1$ $x_3 = +1$	-9.61	
		$x_2 = +1$ $x_3 = -1$	12.44	
2 nd Case: $x_1 = -1$; $x_4 = -1$; $x_5 = -1$				
$\mu\text{g GFP}_{uv}/\text{mL} = 2.58 + 5.38\text{ }x_2 - 5.65\text{ }x_3$				
		$x_2 = +1$ $x_3 = +1$	2.32	1
		$x_2 = -1$ $x_3 = -1$	2.84	
		$x_2 = -1$ $x_3 = +1$	-8.45	
		$x_2 = +1$ $x_3 = -1$	13.61	
3 rd Case: $x_1 = +1$; $x_4 = -1$; $x_5 = -1$				
$\mu\text{g GFP}_{uv}/\text{mL} = 3.84 + 5.39\text{ }x_2 - 5.65\text{ }x_3$				
		$x_2 = +1$ $x_3 = +1$	3.58	5
		$x_2 = -1$ $x_3 = -1$	4.10	
		$x_2 = -1$ $x_3 = +1$	-7.19	
		$x_2 = +1$ $x_3 = -1$	14.87	
4 th Case: $x_1 = -1$; $x_4 = +1$; $x_5 = -1$				
$\mu\text{g GFP}_{uv}/\text{mL} = 0.16 + 5.38\text{ }x_2 - 5.65\text{ }x_3$				
		$x_2 = +1$ $x_3 = +1$	-0.10	
		$x_2 = -1$ $x_3 = -1$	0.42	
		$x_2 = -1$ $x_3 = +1$	-10.87	
		$x_2 = +1$ $x_3 = -1$	11.19	
5 th Case: $x_1 = 0$; $x_4 = 0$; $x_5 = -1$				
$\mu\text{g GFP}_{uv}/\text{mL} = 2.00 + 5.38\text{ }x_2 - 5.65\text{ }x_3$				
		$x_2 = 0$ $x_3 = 0$	2.00	
		$x_2 = +1$ $x_3 = +1$	1.74	
		$x_2 = -1$ $x_3 = -1$	2.26	
		$x_2 = -1$ $x_3 = +1$	-9.03	
		$x_2 = +1$ $x_3 = -1$	13.03	

Table 4 – Fitted models for the expression of GFPuv ($\mu\text{g GFPuv/ mL}$) by setting up levels for the factors: x_1 = storage at 4°C; x_2 = rotary speed (RPM); x_3 = addition of IPTG at OD₆₆₀ (OD₆₆₀ IPTG); x_4 = final concentration of IPTG (mM), with the variable x_5 = +1, incubation set at 37°C for 24 h.

$\mu\text{g GFP}_{uv}/\text{mL} = 10.28 - 1.13\text{ }x_1 - 5.65\text{ }x_3 + 8.28\text{ }x_5 - 1.76\text{ }x_1x_5 - 5.38\text{ }x_2x_5 + 1.21\text{ }x_4x_5$				
FACTOR RELATION	FITTED EQUATION ($\mu\text{g GFP}_{uv}/\text{mL}$)	Extreme Conditions	$\mu\text{g GFP}_{uv}/\text{mL}$	ASSAYS
1 st Case: $x_1 = +1$; $x_4 = +1$; $x_5 = +1$				
$\mu\text{g GFP}_{uv}/\text{mL} = 16.88 - 5.38\text{ }x_2 - 5.65\text{ }x_3$		$x_2 = +1\text{ }x_3 = +1$	5.85	3
		$x_2 = -1\text{ }x_3 = -1$	27.90	
		$x_2 = -1\text{ }x_3 = +1$	16.61	
		$x_2 = +1\text{ }x_3 = -1$	17.14	
2 nd Case: $x_1 = -1$; $x_4 = -1$; $x_5 = +1$				
$\mu\text{g GFP}_{uv}/\text{mL} = 20.23 - 5.38\text{ }x_2 - 5.65\text{ }x_3$		$x_2 = +1\text{ }x_3 = +1$	9.20	13, 14, 15
		$x_2 = -1\text{ }x_3 = -1$	31.26	
		$x_2 = -1\text{ }x_3 = +1$	19.97	
		$x_2 = +1\text{ }x_3 = -1$	20.49	
3 rd Case: $x_1 = +1$; $x_4 = -1$; $x_5 = +1$				
$\mu\text{g GFP}_{uv}/\text{mL} = 14.45 - 5.38\text{ }x_2 - 5.65\text{ }x_3$		$x_2 = +1\text{ }x_3 = +1$	3.42	
		$x_2 = -1\text{ }x_3 = -1$	25.48	
		$x_2 = -1\text{ }x_3 = +1$	14.19	
		$x_2 = +1\text{ }x_3 = -1$	14.71	
4 th Case: $x_1 = -1$; $x_4 = +1$; $x_5 = +1$				
$\mu\text{g GFP}_{uv}/\text{mL} = 22.65 - 5.38\text{ }x_2 - 5.65\text{ }x_3$		$x_2 = +1\text{ }x_3 = +1$	11.62	1, 2
		$x_2 = -1\text{ }x_3 = -1$	33.68	
		$x_2 = -1\text{ }x_3 = +1$	22.39	
		$x_2 = +1\text{ }x_3 = -1$	22.92	
5 th Case: $x_1 = 0$; $x_4 = 0$; $x_5 = +1$				
$\mu\text{g GFP}_{uv}/\text{mL} = 18.55 - 5.38\text{ }x_2 - 5.65\text{ }x_3$		$x_2 = 0\text{ }x_3 = 0$	18.55	
		$x_2 = +1\text{ }x_3 = +1$	7.52	
		$x_2 = -1\text{ }x_3 = -1$	29.58	
		$x_2 = -1\text{ }x_3 = +1$	18.29	
		$x_2 = +1\text{ }x_3 = -1$	18.81	

Figure 1. Graphed data of cell densities (OD_{660}) and dry cell weight ("DCW") correlated with the amount of *GFPuv* ($\mu\text{g GFPuv/mL}$) expressed during 24h *E. coli* growth, as measured by fluorescence intensity of extracted *GFPuv*.

Figure 2. Surface response representing effects of incubation (x_5) at 37°C, previous storage at 4°C and IPTG concentration added at variable OD_{660} densities over the expression of *GFPuv* from *E. coli* cells cultivated at variable speed agitations:

- (i) incubation for 8 h with storage for 48 h and 0.05mM IPTG addition;
- (ii) incubation for 24 h with storage for 24 h and 0.5mM IPTG addition;
- (iii) incubation for 24 h with storage for 48 h and 0.05mM IPTG addition.

Incubation 24h ($x_5=+1$), storage 24h/4C ($x_1=-1$), 0.5mM IPTG ($x_4=+1$)

(ii) $\mu\text{g GFPuv/mL} = 22.65 - 5.38 x_2 - 5.65 x_3$

Incubation 24h ($x_5=+1$), storage 48h/4C ($x_1=+1$), 0.05mM IPTG ($x_4=+1$)

(iii) $\mu\text{g GFPuv/mL} = 14.45 - 5.38 x_2 - 5.65 x_3$

Anexo 07

“Evaluation of Recombinant Green Fluorescent Protein (*GFPuv*) Purification with Various “HiTrap” HIC Media”

Trabalho apresentado na forma de poster no
25th Symposium on Biotechnology for Fuels and Chemicals,
Colorado, Maio de 2003

O propósito deste trabalho foi selecionar o meio de cromatografia de interação hidrofóbica (HIC) mais apropriado para a separação da *GFPuv* do extrato de células.

A seleção da coluna de HIC foi realizada por análise qualitativa comparativa da eletroforese SDS-PAGE de amostras de *GFPuv* coradas com Coomassie Blue ou nitrato de prata.

Research note

Evaluation of Recombinant Green Fluorescent Protein (GFP_{uv}) Purification with Various “HiTrap” HIC Media

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SUMMARY

Transformed cells of *Escherichia coli* expressing recombinant green fluorescent protein (*GFPuv*) were harvested with the centrifuged pellets resuspended in extraction buffer and subjected to the three-phase partitioning (TPP) extraction method. Protein extracts were eluted through one of four types of “Hi-Trap” 1 mL columns, pre-packed with hydrophobic interaction chromatography (HIC) media composed of methyl, butyl, octyl and phenyl resins. The fluorescence intensity (excitation/emission maxima at 394/509nm) of the eluted samples were related to μg *GFPuv*/mL, and analyzed further on a 12% SDS-polyacrylamide gel. With Coomassie staining, a single band between 27 kDa (standard *GFPuv*) and 29 kDa (molecular weight standard) was visualized for every HIC column sample, verifying the effectiveness of TPP-extraction. However, it was not possible to determine by SDS-PAGE which HIC column further improved the purity of the extracted *GFPuv*. The more sensitive silver staining showed several bands greater than 29 kDa from the butyl HIC eluted samples, suggesting it did not optimally purify *GFPuv*. The recovery and purification of *GFPuv* with TPP-extraction and elution through the selected methyl HIC columns was shown to yield very high yields; around 90.10 ± 4.11 % of *GFPuv* recovered and 8-fold of enrichment of *GFPuv* related to the specific mass.

Key words: Recombinant green fluorescent protein, *GFPuv*; hydrophobic interaction chromatography (HIC); SDS-PAGE electrophoresis, Three-phase partitioning (TPP), extraction, purification, protein.

Introduction

The recombinant green fluorescent protein, *GFPuv* (excitation and emission maxima at 394nm and 509nm) expressed by *E. coli DH5-α*, exhibits fluorescence intensity 18 times greater than the wild-type GFP protein of the jellyfish *Aequorea victoria*. *GFPuv* is a compact, globular acidic protein (pI 4.6 to 5.4) consisting of 238 amino acids, a monomer with molecular weight between 27 - 29 kDa that has a propensity to dimerize (1).

Manipulating the expression of a protein can provide a powerful tool for understanding its biological characteristics and potential applications (2). *GFPuv* has proven to be an excellent marker due to its facility to be expressed by various organisms. The fluorescence of *GFPuv* requires no co-factor and is the preferred genetic marker because it can be easily monitored in a wide variety of applications.

Since *GFPuv* exhibits stability to extreme conditions such as exposure to heat and chemical denaturants (disinfectants) in a wide pH range. Its expression by prokaryotes, followed by extraction and purification, should be studied for its potential utility as a marker in validation procedures. In addition, the protein extracted from *E. coli* and further purified should be analyzed qualitatively by SDS-PAGE to define the best extraction-separation method.

Hydrophobic interaction chromatography (HIC) media are used for separating proteins from complex mixtures by their degree of hydrophobic properties (3). The selection of the most appropriate HIC media for the purification of *GFPuv* should be determined through a simultaneous comparison of protein samples run on gel electrophoresis. SDS-PAGE with Coomassie or silver staining provides a

sensitive method to determine the most appropriate HIC support for the purification of *GFPuv*.

The aim of this work was to select the most appropriate HIC media for the separation of TPP-extracted *GFPuv* from total cell extract (expressed by bovine serum albumin) by analyzing the purity of *GFPuv*. The selection of HIC column was performed with comparative qualitative analysis with SDS-PAGE and post-electrophoresis staining with either Coomassie or silver.

Material and Methods

Transformation of E. coli cells; expression and extraction of the recombinant green fluorescent protein (GFPuv): *Escherichia coli* DH5- α (3,6) was transformed with a high copy plasmid, pGFPuv (Clontech, CA, USA), by the standard calcium chloride method (4). Cultures were transferred to a 250 mL Erlenmeyer and cultivated in 50 mL Luria Bertani broth (LB, USB, Mexico) supplemented with 100 μ g/mL ampicilin (Boehringer, Mannheim, Germany) and incubated (37°C/24 h/100 rpm). At an OD₆₆₀ around 0.8, IPTG (isopropyl- β -D-thiogalactopyranoside, USB, Italy) was added to a final concentration of 0.5 mM (5). The *GFPuv* expression by induced cells was accompanied every hour by UV light at $\lambda = 395$ nm. For all cultures, the OD₆₆₀ with LB/amp broth in the reference cell was read and the cellular concentration was obtained by the gravimetric method of the biomass (mg/L) held on the surface of a 0.22 μ m membrane (Millipore, SP, Br) and submitted to 105°C around 24h, attaining constant weight. The biomass concentration related to isolated

GFPuv was expressed by specific productivity ($\mu\text{g GFPuv/ mg DCW}$, DCW = dried cellular weight).

Extraction of GFPuv by three- phase- partitioning extraction (TPP) method (5, 6, 7). The TPP method was applied to induced *E. coli* cultures over-expressing *GFPuv*. Through the TPP extraction, proteins (other than *GFPuv*), nucleic acids and other molecules were precipitated and separated from *GFPuv*. At the end of 24 h incubation, the cultures were centrifuged ($1000\times g/30 \text{ min}/4^{\circ}\text{C}$) and the pellets resuspended into 4 mL cold extraction buffer (XE: 25 mM Tris-HCl, pH 8.0 - Trizma® Base, Sigma, MO, USA; 1.0 mM β -mercaptoethanol " β -ME", Amersham Biosciences, Sweden; 0.1 mM phenylmethylsulphonylfluoride "PMSF", USB, Switzerland). From every suspension in XE, each of eight aliquots of 450 μL was subjected to one-step extraction by the three-phase-partitioning (TPP) method.

To each 450 μL of cell suspension, 300 μL of 4 M $(\text{NH}_4)_2\text{SO}_4$ (1.6 M final concentration) and 750 μL of *t*-butanol (ratio 1:1) were added. The mixtures were vortexed for 1.0 min, centrifuged at $6,000 \text{ g}/10 \text{ min}$. The three phases formed were collected. After the *t*-butanol upper layer and the white interfacial precipitate were removed, another equal volume of *t*-butanol was mixed with the lower aqueous layer and centrifuged. The upper layer of the system was discarded. The interfacial green layer was collected and dissolved in 1.0 mL XE buffer. Every eight TPP-extracted aliquots from the same pellet cell culture were pooled. The final concentration of *GFPuv* into TPP extraction-aliquots varied up to $50 \mu\text{g GFPuv/ mL}$ (Table 2), for a mean specific productivity of $25.30 \mu\text{g GFPuv/ mg DCW}$.

Purification of GFPuv through Five HIC columns: Protein extracts were eluted through five “Hi-Trap” 1 mL columns, pre-packed with four hydrophobic interaction chromatography (HIC) media: methyl support (Macro-Prep HIC supports, Bio-Rad, CA, USA); the Hi-Trap HIC fast flow (FF) columns (Amersham Biosciences, NJ, USA) butyl 4 FF; octyl 4 FF; phenyl 6 FF (low sub) sepharose. From every pelleted cell culture, aliquots of 250 μ L TPP-extract were mixed with 250 μ L 4 M $(\text{NH}_4)_2\text{SO}_4$ and transferred to the top of each of the four fast flow support. The column was previously equilibrated with 2 M $(\text{NH}_4)_2\text{SO}_4$. After the column was loaded, *GFPuv* was retained near the top of the column by affinity binding to the HIC resin. The loaded column was washed first with 250 μ L 1.3 M $(\text{NH}_4)_2\text{SO}_4$ to elute proteins other than *GFPuv* that bind with low affinity. *GFPuv* was eluted with 750 μ L of buffer solution (10 mM Tris-HCl, 10 mM EDTA, pH 8.0), and stored at 4°C. The progress of *GFPuv* through the column was observed with a UV light, as well by the analysis of the eluted material. The fluorescence intensity (excitation/emission maxima at 394/509nm) of eluted samples were related to μ g *GFPuv*/mL by the standard (1) curve.

Electrophoresis (8). The eluted *GFPuv* samples were run on a 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) along with samples of TPP-extracted *GFPuv*. The first 30 minutes was performed at a fixed voltage of 50 V, increased to 200 V for the last 40 min. The protein bands were visualized with Coomassie Brilliant Blue (CBB). The same samples were run later on another 12% SDS polyacrylamide gel at a fixed voltage of 350V (~1hour), and stained with silver nitrate solution, by the PhastSystem (Amersham Biosciences, USA).

The fluorescence intensity of GFPuv was measured in the eluted samples in the spectrofluorophotometer (RF-5301 PC – Shimadzu, Kyoto, Japan), with an excitation filter of 394 nm and an emission filter of 509 nm. The known amounts of purified recombinant *GFPuv* (“standard *GFPuv*”, Clontech®) diluted in buffer (10 mM Tris-HCl, pH 8.0, 1.0 mM β ME, 0.1 mM PMSF) were used to generate a standard curve (1) ($\mu\text{g GFPuv/ mL} = 0.0256 \times (\text{fluorescence intensity}) + 0.8576$; $R^2 = 0.99$), in order to relate the protein concentration with fluorescence intensity of the TPP extracted/ HIC purified aliquots.

The total protein concentration released (5) in the eluted samples was compared relative to purified bovine serum albumin in buffer solution (BSA, mol wt. 66 kDa, Sigma, MO, USA) at A_{280} in a spectrophotometer, and expressed in mg BSA/mL. The total protein concentrations in the buffer solution ranged from 100 to 1000 $\mu\text{g/mL}$ with the maximum $A_{280} = 0.615$. The relationship between total proteins and BSA was made through the standard curve ($\mu\text{g BSA/mL} = 1727.2 \times (A_{280}) - 26.863$; $R^2 = 0.99$). The specific *GFPuv* mass was expressed by $\mu\text{g GFPuv/ mg BSA}$.

Results

Separation efficiency of the Hydrophobic Interaction Chromatography Supports (HIC) on GFPuv purification

Hydrophobic interaction chromatography (HIC) is a technique for separating biomolecules from complex sample mixtures by their degree of hydrophobicity (3, 9). The selected four different media are pre-packed in ready-to-use 1 mL Hi-Trap®

columns, which consist of HIC supports with similar hydrophobic characteristics, appropriate for purification of intermediate to weakly hydrophobic proteins (9). The selection of these various resins allowed for screening the most appropriate HIC support for the purification of the TPP-extracted *GFPuv* from a complex mixture of proteins typically found in cell extracts.

HIC is ideally suitable for application after salt precipitation of the TPP-extracted *GFPuv* from the pelleted cells, since HIC uses a decreasing salt gradient as the mobile phase, preserving the fluorescence of *GFPuv*. Ammonium sulfate is the most commonly used salt in both TPP-extraction and HIC methods (3, 7, 9).

Electrophoresis analyzes of the eluted GFPuv samples.

TPP-extracted *GFPuv* from pelleted cells of *E. coli* were eluted through four Hi-Trap fast flow HIC columns, methyl; butyl; octyl; phenyl sepharose. The fluorescence intensity (excitation/emission maxima at 394/509nm) of eluted samples were related to $\mu\text{g GFPuv/mL}$ and then, the same samples were run on a 12% SDS-PAGE (Figures 1, 2).

A single band between 27 kDa (standard *GFPuv*) and 29 kDa (standard molecular weight) for every HIC column sample (Figure 1) was visualized by Coomassie staining solution. Using the more sensitive silver staining, the butyl HIC eluted samples showed several bands greater than 29 kDa (Figure 2), showing that this media is not optimal for further purification of *GFPuv*. As seen in every lane, the gels proved the effectiveness of TPP-extraction for *GFPuv* and the bands observed were similar for every sample eluted through a column. However, with SDS-PAGE it was not possible to distinguish which HIC column improved the purity of the extracted *GFPuv*.

Quantification of Total Proteins (BSA) in Eluted Samples

Through Table 1, we observe that the amount of BSA eluted with *GFPuv* from the methyl and butyl HIC columns were half those recovered in the samples eluted from the octyl and the phenyl columns, for a range of respective mean concentrations of *GFPuv* from 27.92 $\mu\text{g/mL}$ (methyl column) to 28.66 $\mu\text{g/mL}$ (phenyl support), corresponding to a total average of $28.25 \pm 0.36 \mu\text{g GFPuv/mL}$.

The same HIC column was used five times with five different samples from the same TPP-extracted 4 mL-aliquot. The *GFPuv* concentration, eluted from the four columns and from five runs through each of the columns, was shown to be equivalent for all four HIC supports. This efficiency was confirmed with SDS-PAGE and Coomassie staining (Figure 1). In relation to the removal of BSA from the eluted sample, for the first, second and third runs, the butyl column provided the higher specific masses; and for the fourth and fifth runs the methyl support increased the specific mass up to 138-78.95 $\mu\text{g GFPuv/mg BSA}$, confirming the heterogeneity of different materials present in the aliquots before elution through the HIC media. Otherwise, the SDS-PAGE and silver staining (Figure 2) showed that the butyl HIC eluted samples showed several bands greater than 29 kDa, showing that this media is not optimal for the further purification of *GFPuv*. The use of the same HIC columns after five elutions of the TPP-extracted aliquot did not interfere in the evaluation of their performances.

For further work, the methyl HIC column was chosen for the purification of the TPP-extracted *GFPuv* samples. The *GFPuv* recovery and enrichment from the eluted samples were also determined (Table 2).

Hi-Trap HIC selection for GFPuv purification

The Macro-Prep HIC methyl (weakly hydrophobic) support are rigid acrylic beads for small scale purification of biomolecules, while the HIC media for the other supports are based on the highly cross-linked beaded agarose matrix, Sepharose^{TM} Fast Flow.

Butyl, Octyl and Phenyl Fast Flow supports are applied for initial and intermediate step purifications of proteins that require a matrix with low to medium hydrophobicity. Octyl Sepharose Fast Flow is also used as an important complement to the other hydrophobic matrices.

Butyl Sepharose Fast Flow often works efficiently with low salt concentrations, which explains the several bands with molecular weight greater than 29 kDa (*GFPuv* molecular weight) separated by the SDS-PAGE with silver staining (Figure 2). The mechanism of adsorption and desorption onto the butyl surface is different from that onto the other matrices, explaining the extra protein bands observed by electrophoresis.

The type of surface determines primarily the selectivity of the HIC adsorbent. In general, HIC media fall into two groups depending on their interactions with sample components. Straight alkyl chains (methyl, butyl, octyl) show a “pure” hydrophobic character, while aryl ligands (phenyl) show mixed performances, where both aromatic and hydrophobic interactions, as well as lack of charge, play simultaneous roles. This special property of the phenyl support explains the high selectivity for materials (expressed by 3.18 mg BSA/ mL, Table 1) other than *GFPuv* through the first elution of the phenyl support, with the affinity changed over subsequent elutions.

The methyl HIC support was selected among the four studied matrixes for the final purification step of TPP-extracted *GFPuv* because it exhibited convenient performance. Furthermore, the butyl column provided inadequate purification and the phenyl media properties changed after subsequent multiple elutions.

Enrichment and Recovery of GFPuv in eluted samples from methyl columns

The number of hydrophobic groups covering the surface of the methyl matrix is higher than the other matrices, resulting in a higher capacity for protein retention. Although the protein may contain a large percentage of hydrophobic residues throughout its structure, only residues at the surface contribute to the hydrophobic character of the native protein.

The purification efficiency of *GFPuv* eluted from the methyl HIC columns was evaluated through (i) the enrichment (x, times) and (ii) recovery (%) indices (7, 9).

(i) Enrichment (x):

$$\text{Enrichment} = \frac{\text{specific mass of the eluted purified } GFPuv \text{ sample}}{\text{specific mass of the TPP-extracted } GFPuv \text{ aliquot}}$$

The enrichment measures the number of times the eluted sample was further purified in terms of *GFPuv* concentration relative to the reduction of the total protein (BSA) concentration, and maintaining the amount of the TPP-extracted *GFPuv* in the aliquot before elution through the columns.

The “Enrichment” index measures the level of purity of the *GFPuv* samples by the increase of the specific mass ($\mu\text{g } GFPuv / \text{mg BSA}$) due to the removal of

proteins (quantified as BSA) other than *GFPuv* (5, 8). Enrichment is expressed in terms of number of times (x) the specific mass of the eluted purified *GFPuv* sample ($\mu\text{g } GFPuv / \text{mg BSA}$) is greater than the corresponding specific mass of the TPP-extracted *GFPuv* aliquot ($\mu\text{g } GFPuv / \text{mg BSA}$) before HIC, by the reduction of BSA concentration and maintaining the initial concentration of *GFPuv*.

(ii) Recovery (%):

$$\text{Recovery} = \frac{\text{eluted } GFPuv \text{ sample } (\mu\text{g } GFPuv / \text{mL})}{^1\text{TPP-extracted } GFPuv \text{ aliquot}} \times 100\%$$

Note: ¹ 1mL aliquot of TPP-extracted *GFPuv* loaded onto the HIC column.

The “Recovery” index measures the percentage (%) of the amount of TPP-extracted *GFPuv* that was present in the purified *GFPuv* sample after passage through the HIC columns.

Each TPP-extracted aliquot was divided in two samples that were independently loaded onto the methyl support (Table 2), providing a total of 64 eluted samples in duplication: first group of 32 samples plus second group of 32 samples.

The recovery index varied from 72.67% to 107.13% and the methyl support showed a very high efficiency in the recovery of *GFPuv*. From 64 assays, 7 samples of the first group and 7 samples of the second group presented recovery of *GFPuv* between 70% and 80%. The majority of the samples (around 78%) showed a recovery index greater than 80% of *GFPuv* in the eluted samples. Therefore, the range for *GFPuv* recovery index from the eluted samples was from (90.88%±3.62) for the first group to (89.32±4.60) for the second group.

Even for samples with concentrations lower than 10 $\mu\text{g GFPuv/mL}$ (from 1.15 to 8.18 $\mu\text{g GFPuv/mL}$, in 7 TPP-extracted aliquots), the recovery was confirmed to be higher than 80%.

On the other hand, the enrichment of *GFPuv* that was given by the relation between the specific mass of the eluted sample and the TPP-extracted aliquot, showed to be dependent upon the quality and amount of the material (determined with BSA) that was carried out in the TPP-extracted aliquots and or mixed with *GFPuv*. The enrichment index varied from: (i) 1.17-2.05 times for a 5.33 $\mu\text{g GFPuv/mL}$ aliquot corresponding to (ii) 24.67-28.36x for 1.15 $\mu\text{g GFPuv/mL}$ and 22.14 $\mu\text{g GFPuv/mL}$ TPP-extracted aliquots respectively. Each 25 eluted samples of the first plus the second groups had their specific mass enriched up to 9.5-fold. Otherwise, a total of 12 eluted samples belonging to the first group (six samples) and the second group (six samples) provided *GFPuv* enrichment between 10 and 20-fold, and one sample of each group showed 28 and 24-fold enrichment, respectively. Therefore, the average index of enrichment provided by the methyl HIC support over TPP-extracted aliquots ranged from 8.46 ± 2.13 increase (first group) to 7.60 ± 2.00 increase (second group).

Taking into consideration the total proteins in the samples, expressed in mg BSA/ mL, three and four eluted samples of first and second groups, respectively, contained concentrations greater than 0.5 mg BSA/mL, and three samples of the first group had up to 0.7 mg BSA/mL, independent on the initial specific mass of the TPP-aliquot eluted through the methyl HIC media (Tables 1, 2). This observation reinforces the different characteristics of the material represented by BSA that was extracted with *GFPuv* from the pelleted cells. Therefore, the HIC procedure did

improve TPP effectiveness on the *GFPuv* purification, confirming Sharma's (7, 11) and Yakhinin's (10) observations.

Conclusions

The effectiveness of the TPP-extraction of *GFPuv* from *E. coli* was analyzed by electrophoresis and confirmed to be the same in every lane for samples eluted from all HIC columns. The performance of the HIC columns was not affected after five elutions per column.

The enrichment in *GFPuv* in the eluted samples was dependent upon the amount of total proteins (expressed in mg BSA/mL). After elution through the methyl HIC columns, the recovery and the enrichment of *GFPuv* in the eluted samples exhibited very high indices, around 90.10 ± 4.11 % of *GFPuv* recovery and 8.03 ± 2.07 -fold enrichment of *GFPuv* related to the specific mass.

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Table 1. Protein concentration in the eluted samples from the HIC columns expressed in terms of recombinant green fluorescent protein ($\mu\text{g GFPuv}/\text{mL}$), bovine serum albumin (BSA, $\text{mg BSA}/\text{mL}$) and specific mass ("SM"; $\mu\text{g GFPuv}/\text{mg BSA}$). The range of *GFPuv* concentration analyzed was around 20-30 $\mu\text{g}/\text{mL}$.

HIC Column	Protein Concentration	Eluted Samples				
		1	2	3	4	5
Methyl	<i>GFPuv</i> ($\mu\text{g}/\text{mL}$)	28.33	27.13	26.58	28.27	29.31
	BSA (mg/mL)	1.65	0.45	0.49	0.20	0.37
	SM ($\mu\text{g}/\text{mg}$)	17.12	59.98	54.60	138.35	78.95
Butyl	<i>GFPuv</i> ($\mu\text{g}/\text{mL}$)	27.72	23.78	29.89	29.06	31.85
	BSA (mg/mL)	0.87	0.25	0.33	0.26	1.09
	SM ($\mu\text{g}/\text{mg}$)	31.85	96.00	90.30	110.69	29.12
Octyl	<i>GFPuv</i> ($\mu\text{g}/\text{mL}$)	27.56	25.37	29.05	29.62	28.27
	BSA (mg/mL)	2.63	0.27	1.01	0.60	0.43
	SM ($\mu\text{g}/\text{mg}$)	10.50	95.52	28.78	49.00	65.34
Phenyl	<i>GFPuv</i> ($\mu\text{g}/\text{mL}$)	22.92	25.93	30.50	31.07	32.88
	BSA (mg/mL)	3.18	0.39	0.76	1.05	0.44
	SM ($\mu\text{g}/\text{mg}$)	7.22	65.87	40.05	29.46	74.01

Table 2. Enrichment and recovery of TPP-extracted *GFPuv* aliquots in eluted samples from methyl HIC columns.

¹ TPP-extracted <i>GFPuv</i> aliquot				Eluted Samples from Methyl HIC columns									
				First Group					Second Group				
<i>GFPuv</i> ($\mu\text{g/mL}$)	BSA (mg/mL)	SM ($\mu\text{g/mg}$)	<i>GFPuv</i> ($\mu\text{g/mL}$)	BSA (mg/mL)	SM ($\mu\text{g/mg}$)	<i>GFPuv</i> (times)	<i>GFPuv</i> (%)	<i>GFPuv</i> ($\mu\text{g/mL}$)	BSA (mg/mL)	SM ($\mu\text{g/mg}$)	<i>GFPuv</i> (times)	<i>GFPuv</i> (%)	<i>GFPuv</i> (%)
1.15	4.79	0.24	1.10	0.29	3.84	16.02	95.52	1.19	0.20	5.91	24.67	103.40	
1.21	0.89	1.37	1.13	0.15	7.57	5.54	92.88	1.21	0.48	2.51	1.84	99.70	
2.03	1.74	1.16	1.47	0.41	3.60	3.09	72.72	1.62	0.53	3.04	2.61	80.05	
3.65	3.72	0.98	3.29	0.71	4.65	4.75	90.27	3.67	0.54	6.83	6.98	100.57	
5.33	0.81	6.56	4.51	0.34	13.46	2.05	84.63	5.03	0.65	7.70	1.17	94.38	
6.86	0.77	8.89	6.68	0.20	33.67	3.79	97.50	6.82	0.36	18.90	2.12	99.43	
8.18	1.89	4.33	8.28	0.24	35.12	8.11	101.22	8.71	0.43	20.39	4.71	106.50	
10.17	3.40	2.99	9.82	0.53	18.51	6.20	96.60	10.27	0.45	22.89	7.66	100.97	
11.30	2.91	3.89	10.99	0.40	27.75	7.14	97.29	10.94	0.33	33.40	8.59	96.88	
12.56	0.90	13.97	14.33	0.36	40.32	2.89	108.36	12.82	0.51	24.91	1.78	102.05	
15.50	5.56	2.79	11.43	0.24	46.72	16.77	73.73	11.53	0.29	39.81	14.29	74.40	
17.35	1.79	9.72	18.59	0.31	59.22	6.10	107.13	11.93	0.49	24.28	2.50	68.77	
19.79	4.70	4.21	15.98	0.29	55.91	13.28	72.67	15.08	0.60	25.21	5.99	76.20	
20.09	3.49	5.75	20.41	0.62	32.82	5.71	101.61	16.96	0.36	47.55	8.27	84.41	
20.80	4.83	4.30	16.48	0.31	52.54	12.21	79.26	15.69	0.45	34.55	8.03	75.46	
21.31	2.22	9.58	22.33	0.58	36.27	3.99	99.56	17.60	0.50	35.22	3.67	82.60	
22.13	7.92	2.79	17.27	0.70	24.70	8.84	74.16	17.66	0.47	37.36	13.38	75.79	
22.14	9.53	2.32	17.86	0.27	65.88	28.36	80.67	16.26	0.53	30.40	13.09	73.45	
23.76	2.49	9.55	18.83	0.36	53.00	5.55	79.25	19.58	0.32	61.89	6.48	82.43	
26.66	4.43	6.02	21.36	0.49	43.64	7.25	80.13	21.18	0.56	37.77	6.28	79.43	
27.07	5.70	4.75	27.06	0.28	95.82	20.17	89.96	38.83	0.45	85.45	17.99	136.25	
27.83	4.32	6.44	29.52	0.35	84.87	13.18	100.78	25.67	0.24	105.86	16.43	92.24	
28.51	2.07	13.80	26.35	0.48	54.35	3.94	92.43	22.72	0.42	54.58	3.96	79.69	
28.82	1.99	14.47	25.53	0.55	46.12	3.19	79.74	24.68	0.67	36.96	2.55	85.67	
29.26	1.34	21.86	27.30	0.37	74.74	3.42	93.31	27.15	0.44	61.75	2.82	92.81	
29.81	2.41	12.35	27.01	0.32	84.48	6.84	90.64	24.01	0.39	62.10	5.03	80.56	
31.49	6.16	5.11	31.17	0.38	81.39	15.91	99.00	27.95	0.36	78.66	15.38	88.75	
31.90	8.23	3.88	28.90	0.44	65.78	16.97	90.57	27.59	0.47	58.76	15.16	86.49	
32.85	1.62	20.25	30.31	0.37	82.80	4.09	92.27	28.01	0.35	79.81	3.94	85.27	
34.13	4.28	7.97	31.66	0.55	57.33	7.19	88.12	29.14	0.60	48.66	6.10	85.36	
38.34	2.85	13.47	39.46	0.74	53.33	3.96	102.92	37.28	0.39	95.39	7.08	97.23	
42.60	1.56	27.39	43.97	0.37	119.72	4.37	103.23	38.83	0.54	71.95	2.63	91.16	

Legend:

¹TPP-extracted GFPuv aliquot before HIC fast flow

SM = Specific Mass (μg GFPuv/ mg BSA)

GFPuv (%) = $\text{Recuperation (\%)} = (\text{eluted GFPuv sample} / \text{TPP-extracted GFPuv aliquot}) * 100$

GFPuv (times) = $\text{Enrichment (x)} = (\text{specific mass of eluted GFPuv sample} / \text{specific mass of TPP-extracted GFPuv aliquot})$

Figure 1. Polyacrylamide gel run by SDS-PAGE. Protein was stained with Coomassie Brilliant Blue. The eluted samples from HIC columns applied to the wells were: 1st lane-mass molecular weighted 18 to 200 kDa (Gibco), 2nd lane-standard *GFPuv* (20 $\mu\text{g/mL}$), 3rd lane-sample eluted from Methyl column (0.32 μg); 4th lane-sample eluted from Butyl column (0.30 μg); 5th lane-sample eluted from Octyl-column (0.29 μg); 6th lane--sample eluted from (0.26 μg).

Figure 2. . Polyacrylamide gel run by SDS-PAGE (PhastSystem). Protein was stained with silver nitrate. The gel visualization was altered by nitrate oxidation. The eluted samples from HIC columns applied to the wells corresponded to: 1st lane-mass molecular weight markers, 18 to 200 kDa (Gibco), 2nd lane-sample eluted from Methyl column (0.085 μ g); 3rd lane-sample eluted from Butyl column (0.080 μ g); 4th lane-sample eluted from Octyl- column (0.077 μ g); 5th lane-sample eluted from Phenyl column (0.069 μ g)

Anexo 08

“Thermal characteristics of recombinant green fluorescent protein (*GFPuv*) extracted from *Escherichia coli* cells”.

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Este trabalho visou o estudo da termoestabilidade da proteína verde fluorescente recombinante *GFPuv* em diferentes temperaturas sob seu pH ótimo 8.0, visando a sua utilização como indicador biológico de rápida resposta em tratamentos térmicos com vapor ($T < 100^{\circ}\text{C}$).

A comparação foi realizada entre a proteína padrão *GFPuv* (Clontech®) e a proteína isolada em laboratório.

Conclusões

O estudo dos parâmetros de determinação da resistência térmica da proteína, demonstrou que:

- (i) para processos térmicos à temperatura inferior à 70°C e processos de pasteurização não há alteração na intensidade de fluorescência da *GFPuv* e, (ii) a estabilidade de fluorescência da *GFPuv* começa a ser alterada a temperatura igual ou superior 75°C

A estabilidade da proteína frente a diferentes temperaturas possibilita o seu uso como indicador biológico nos processos de esterilização.

TITLE:

THERMAL CHARACTERISTICS OF RECOMBINANT GREEN
FLUORESCENT PROTEIN (*GFP_{UV}*) EXTRACTED FROM
ESCHERICHIA COLI

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Abbreviated running headline:

THERMAL CHARACTERISTICS OF *GFP_{UV}*

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SUMMARY

Aims: Emulating conditions used for moist heat treatments, the thermal stability of the isolated recombinant green fluorescent protein (*GFPuv*) was evaluated and compared to a standard *GFPuv*, by analyzing the loss of fluorescence intensity.

Methods and Results: Recombinant green fluorescent protein, *GFPuv*, expressed by *E. coli*, isolated by the three-phase partitioning extraction (TPP) method and eluted from an HIC column (isolated *GFPuv*), was diluted in tris-HCl (pH 8.0) and subjected to continuous heating at set waterbath temperatures (45°C to 95°C). The loss of fluorescence intensity (excitation / emission maxima at 394/509nm) was converted to *GFPuv* concentrations (µg/mL). As a determination of the extent of denaturation, decimal reduction times (D-values) for both isolated and standard *GFPuv* were similar and still high at temperatures > 70°C. The mean D-values at 75°C and 95°C were: (i) $D_{75C}=132\text{min}$ and $D_{95C}=2.1\text{min}$ for isolated *GFPuv*, and (ii) $D_{75C}=158.73\text{min}$ and $D_{95C}=4.90\text{min}$ for standard *GFPuv*.

Conclusions: The energy of activation (E_a), in the interval of 75°C to 95°C, ranged from 53.0kcal/mol to 45.5kcal/mol, respectively, for both the isolated and standard *GFPuv*, with a variation of approximately 16%, which is not substantial. Therefore, both systems presented an equivalent thermal stability.

Significance and Impact of the Study: The thermal stability of *GFPuv* provides the basis for its potential utility as a biological indicator, a fluorescent marker to assay the efficacy of low heat treatments with steam.

Key words: thermal stability; decimal reduction time (D-value); biological indicator, sterilization, blanching.

INTRODUCTION

Moist-heat treatments using steam at low temperatures ($T \leq 100^{\circ}\text{C}$) have been applied to a variety of medical, pharmaceutical and food products to hinder microbial growth and inactivate endogenous enzymes to preserve and extend product shelf life. The efficiency of blanching, pasteurization and disinfection is commonly evaluated through the enzymatic activity of the most thermostable enzyme present in the product, which have been observed to exhibit greater thermal stability than contaminating microorganisms (Piyasena *et al.* 1998; Villamiel *et al.* 1997). The selected enzyme is employed as the biological indicator (BI) to evaluate the immediate efficacy of the moist heat procedure and throughout the shelf life of the material. BIs are used to monitor the assurance of satisfactory preservation (Tijssens *et al.* 1999; Eargerman and Rouse 1976; Forsyth *et al.* 1999). Due to the importance of validating the moist-heat treatments and related processes, it is necessary to develop a BI which can efficiently assess the effectiveness of the treatment.

In this work, the use of a fluorescent marker designed for a quick and reliable assay, detectable by microscopy, spectrofluorometry or visual inspection using a hand-held UV lamp, was examined. The recombinant green fluorescent protein (*GFPuv*, excitation/emission maxima at 394/509nm) was shown to exhibit a fluorescence intensity 18 times greater than the wild-type protein [Chalfie *et al.* 1994, Ward 1998]. Recombinant *GFPuv* is a compact, globular acidic protein (pI 4.6 to 5.4) with one fluorophore consisting of a cyclic tripeptide in the primary protein sequence, a chain of 238 amino acids [Cody *et al.* 1993; Ormõ *et al.* 1996; Yang *et*

al. 1996). Functional *GFPuv* is a monomer of 27-29 kDa (Ward 1988) that is exceptionally resistant to heat ($T \geq 70^{\circ}\text{C}$), alkaline pH (between pH 5.5 and 12.0; the optimum pH between 7.0 and 8.0), and some detergents, chaotropic salts and organic solvents (Chalfie *et al.* 1994, Ward 1998).

The purpose of this work was to study the thermal stability of the recombinant protein *GFPuv*, as well as to verify its potential utility as a quick and accurate biological indicator (BI) for moist heat-treatments ($T < 100^{\circ}\text{C}$).

MATERIAL AND METHODS

Transformation and Expression. *Escherichia coli* DH5- α cells (Chalfie 1994) transformed (pGFPuv, Clontech, CA, USA) by the standard calcium chloride method (Sambrook *et al* 1989; Vessoni Penna *et al.* 2002) to express the recombinant green fluorescent protein (*GFPuv*, excitation/emission maxima at 394nm/509nm), were grown (37°C / 100 rpm/ 24h) in Luria Bertani broth (LB, USB, Ohio, USA) supplemented with 100 $\mu\text{g/mL}$ ampicillin (amp, Boehringer, Mannheim, Germany). An aliquot of this culture was transferred onto the surface of LB-amp agar with isopropyl- β -D-thiogalactopyranoside (IPTG) added to final concentration of 0.5 mM (w/v) and incubated at 37°C . After 24h, the agar surface was illuminated with a hand-held UV lamp at 395 nm (Model UVL 4, UVP, CA, USA) and isolated brightly fluorescent colonies were picked and transferred to 25 mL LB-amp broth (pre-inoculum), and incubated (100 rpm/ 37°C) up to an $\text{OD}_{660} = 0.01$ (10^4 CFU/mL); Spectrophotometer, Beckman DU-600, CA, USA). A 1 mL aliquot of this pre-inoculum was transferred into 25 mL LB-amp broth, and the 250 mL flasks were shaken (100 rpm/ 37°C) until the broth cultures attained an $\text{OD}_{660} = 0.8$ (10^7

CFU/mL). IPTG was then added to a final concentration of 0.5 mM. After another 21h (100 rpm/ 37°C), the expression of *GFPuv* by induced cells was detected by illuminating the broth at 395 nm and visually inspecting for intense green fluorescence. Cells were harvested by centrifugation (6.000xg/ 30 min/ 4°C). The supernatant was decanted and the cell pellet was resuspended in 4 mL of chilled (4°C) extraction buffer, ("XE": 25.0 mM Tris-HCl, pH 8.0, 1.0 mM β -mercaptoethanol, " β -ME", Pharmacia Biotech, Sweden; 0.1 mM phenylmethylsulphonylfluoride, "PMSF", USB, Switzerland) prior to subjecting the cells to the three-phase partitioning extraction (TPP) method.

Three-Phase Partitioning Extraction (TPP) Method. (Dennison and Lovrien 1997; Vessoni Penna and Ishii 2002). In a 2 mL microcentrifuge tube, to each aliquot of 450 μ L of resuspended cells, 300 μ L of 4 M $(\text{NH}_4)_2\text{SO}_4$ and 750 μ L of *t*-butanol were added. The mixture was stirred for 2 min at room temperature, centrifuged and the three phases formed were collected separately. The *t*-butanol upper phase and the white interfacial precipitate were removed and discarded. A second aliquot of 750 μ L of *t*-butanol was mixed into the lower aqueous phase. The mixture was allowed to settle to visible phase separation and centrifuged. At the separation of the three phases, the upper phase was discarded. The interfacial green phase was collected and dissolved in 500 μ L XE. While the lower phase was still fluorescent, it was subjected to repeated TPP extraction.

A 1.0 mL aliquot of protein extract was mixed with 1.0 mL 4 M $(\text{NH}_4)_2\text{SO}_4$ and this mixture was transferred to the top of a methyl support hydrophobic interaction column ("HIC"), fast flow, for final purification. The HIC was previously equilibrated with 2 M $(\text{NH}_4)_2\text{SO}_4$. The loaded column was first washed with 250 μ L

1.3 M $(\text{NH}_4)_2\text{SO}_4$ to elute proteins that bind with low affinity to the methylated resin of the column packing. *GFPuv* was eluted with 1.0 mL of buffer solution (10 mM Tris-HCl, 10 mM EDTA, pH 8.0), and stored at 4°C.

The fluorescence intensity (± 0.005) of *GFPuv* was measured in the eluted samples in a fluorescence spectrophotometer (RF 5301 PC, Shimadzu Corporation, Kyoto, Japan), with an excitation filter of 394 nm and an emission filter of 509 nm. Purified recombinant *GFPuv* purchased from Clontech ("standard *GFPuv*"; Clontech, USA) was used to generate a standard curve to determine TPP-extracted *GFPuv* concentration and to provide an experimental comparison for thermal stability. The fluorescence intensity of the TPP-extracted samples were compared with the standard curve: fluorescence intensity (I) = $-33.078 + 38.943 \times (\text{GFPuv } \mu\text{g/mL})$; ($R^2 = 0.997$). To determine the concentration of the TPP-extracted *GFPuv*, fluorescence intensity was correlated to the concentration of native *GFPuv*, since denatured *GFPuv* cannot fluoresce, and the loss in fluorescence intensity was a measure of denatured *GFPuv* concentration.

Denaturation of GFPuv under Heat Treatments. Standard *GFPuv* ("std *GFPuv*") and TPP-extracted *GFPuv* ("isolated *GFPuv*") were each diluted in 1.0 mL buffer solution (10 mM Tris-HCl, 10 mM EDTA, pH 8.0) at concentrations varying between 3.0 and 6.0 $\mu\text{g GFPuv/mL}$. Each sample, in duplicate, was transferred to a quartz cuvette [3 mm x (10 mm light path length) x 45 mm height] and sealed with a plastic cover. Each cuvette was inserted into the adapter assembly and adjusted in the cell holder. The cell holder was connected to a constant-temperature bath and a constant-temperature water circulating device (Thermo-bath TB-85, P/N 200-65022, Shimadzu, Kyoto, Japan) was used to heat and control the temperature of the cell

holder at the assay temperature. The temperature of the waterbath was maintained within $\pm 0.05^\circ\text{C}$. A constant-temperature was provided by continuous circulation of water from the waterbath to the cell holder and the sample in the cuvette via a circulation pump.

From the moment the sample-filled cuvette was placed in cell holder and the heat-treatment was initiated, a fluorescence reading was taken at defined exposure intervals at different temperatures (45°C , 60°C , 65°C , 70°C , 75°C , 80°C , 85°C , 90°C , 95°C ; Tables 1 and 2). The variation in fluorescence intensity (± 0.005) was measured by the spectrofluorometer every 30s and converted to native *GFPuv* concentrations, for initial and final concentrations of $6.0 \mu\text{g/mL}$ and $1.0 \mu\text{g/mL}$, respectively.

Analysis of the Thermal Kinetic Parameters (16). The *GFPuv* inactivation curves were considered first order models represented by $\text{Log}_{10} C_f = \text{Log}_{10} C_o - (1/D)*t = \text{Log}_{10} C_o - (k/2.303)*t$, where C_o was the "initial concentration" of native *GFPuv* ($\mu\text{g/mL}$) and C_f was the "final concentration" of native *GFPuv* ($\mu\text{g/mL}$), after the exposure to the heating time (t , min), D -value, the decimal reduction time (D , h or min) and the inactivation rate constant (k , h^{-1} or min^{-1}). The decimal reduction time (D -value), the interval of time required to reduce one decimal logarithm of the initial fluorescence intensity of *GFPuv*, at reference temperature, was determined from the negative reciprocal of the slopes of the regression lines, using the linear portions of the inactivation curves ($\text{log}_{10} \mu\text{g GFPuv/mL}$ versus time of exposure at a constant temperature). The temperature coefficient, Q_{10} , was calculated to evaluate the dependence of the D -value on an increase of 10°C in the heating temperature (T , $^\circ\text{C}$), by the following relation: $Q_{10} = [D_T/D_{(T+10)}]$. The z -value

(°C), expressed as the number of degrees (°C) required for one Log₁₀ change in the D-value ($D=2.303/k$), was determined by plotting the relation between Log₁₀ D-value and corresponding temperatures. The z-value may be related to the coefficient Q_{10} of the process by: $Q_{10} = (10^{10/z})$. Activation energy (E_a , kcal/mol) was estimated by using the Arrhenius equation, which was applied to evaluate the dependence of the inactivation rate constant “k” on the heating temperature: $\text{Log}_{10} k_1 = \text{Log}_{10} k_2 - \{(E_a / 2.303 \cdot R)[(1/T_1) - (1/T_2)]\}$, where T_1 and T_2 are the heating temperatures in degrees Kelvin (K, $T \text{ (K)} = 273 + T \text{ (°C)}$), and R is the universal gas constant (1.987 calories per mol per K).

RESULTS

Recombinant green fluorescent protein, *GFPuv*, expressed by *E. coli*, isolated by the TPP-extraction method and eluted from an HIC column (isolated *GFPuv*), was diluted in buffer and subjected to continuous heating at set waterbath temperatures. The variation in fluorescence intensity was measured by the spectrofluorometer and converted to *GFPuv* concentrations. After being heated and repeated exposure to light during the assay, the fluorescence intensity of *GFPuv* was constant in samples that were kept at 4°C for 24h. Therefore, no recovery of fluorescent intensity, as may be related to protein renaturation, was recorded.

For temperatures between 45°C and 70°C, the isolated *GFPuv* concentration decreased from 11% to 75% in relation to the initial concentration (Table 1), even after 180 min maximum exposure. For temperatures above 70°C, the average percentage in the *GFPuv* fluorescence decreased from 72% to 83% for minimum of 1.8 min treatment at 95°C and maximum exposure time of 71 min at 75°C. For

standard *GFPuv* subjected to the same heating conditions, the fluorescence decrease of 65-74% was, respectively, at 75°C and 95°C. Post-heating pH did not change and the samples remained clear, without any protein precipitating out of solution.

Spectrum of excitation and emission

The spectral characteristics of the excitation and emission peaks for the samples of *GFPuv* were measured in a spectrofluorometer at room temperature. The excitation and emission spectra were similar for both standard *GFPuv* and isolated *GFPuv* before and after heating, thus confirming that the isolated protein is the *GFPuv* of interest for this experiment and that the extraction by TPP and purification by HIC did not significantly alter the structure of the protein.

Thermal kinetic parameters for the denaturation of GFPuv at different temperatures.

For a comparison between standard and isolated *GFPuv*, the denaturation of protein was measured by the loss of fluorescence intensity and expressed in the decimal logarithm of the decrease in native *GFPuv* concentration versus the time of exposure at constant temperature (Table 1, Figure 1). To estimate D-values (min) by heating at constant temperatures, the interval of *GFPuv* concentration considered was the region corresponding to the linear portion of the inactivation curves ($\text{Log}_{10} \mu\text{g GFPuv} / \text{mL}$ versus time of exposure at a constant temperature; Figure 1). At 95°C, a greater drop in fluorescence intensity is observed for isolated than for standard *GFPuv* (Figure 1). For lower temperatures, the loss of fluorescence intensity decreases gradually.

For isolated *GFPuv*, at temperatures < 75°C (Table 1), the longest D-value of 3,333 min (55.6 hours) was observed at 45°C; and the D-values at 60°C ($D_{60\text{C}} = 720$

min) and 65°C ($D_{65C} = 625$ min) were close (mean $D = 672.5$ min). For an increase of 10°C, the D-value at 60°C was halved at 70°C (mean $D_{70C} = 377.3$ min); and the inactivation rate constant nearly doubled ($Q_{10} = 1.91$). However, the D-value decreased almost 5 to 6-fold in the ranges from 65°C ($D_{65C} = 625$ min) to 75°C (mean $D_{75C} = 132.5$ min) and from 70°C (mean $D_{70C} = 377.3$ min) to 80°C (mean $D_{80C} = 59.2$ min), confirmed by Q_{10} coefficients between 4.7 and 6.38, respectively.

The isolated *GFPuv* exhibited the highest stability at temperatures $\leq 70^\circ\text{C}$; however, the susceptibility of the protein to heat changed at $\geq 75^\circ\text{C}$. From 75°C to 95°C, the protein denaturation rate increased 7-8 times: (i) $Q_{10} = 8.34$, between 75°C (mean $D_{75C} = 132.5$ min) and 85°C (mean $D_{85C} = 15.9$ min); (ii) $Q_{10} = 7.87$, between 80°C (mean $D_{80C} = 59.2$ min) and 90°C (mean $D_{90C} = 7.5$ min); (iii) $Q_{10} = 7.62$, between 85°C (mean $D_{85C} = 15.9$ min) and 95°C (mean $D_{95C} = 2.1$ min).

For temperatures $\geq 75^\circ\text{C}$, standard and isolated *GFPuv* showed close D-values (Table 1, Figure 1) although the D-values for standard *GFPuv* were slightly higher than those for isolated *GFPuv*, respectively: (i) $D_{75C} = 158.7$ min, 1.2 times higher; (ii) $D_{80C} = 71.9$ min, 1.2 times higher, (iii) $D_{85C} = 30.0$ min, 1.8 times higher, (iv) $D_{90C} = 9.8$ min, 1.3 times higher, and $D_{95C} = 4.90$ min, 2 times higher. The stability of both standard and isolated *GFPuv* was still high at temperatures greater than 70°C. For about 2h at a constant 75°C, the D-value was reduced by one log cycle, relating the decrease in fluorescence as an increase in *GFPuv* denaturation.

The kinetic parameters z-value and the related Q_{10} coefficient ($Q_{10} = 10^{10/z}$) reflect the temperature dependence of the denaturation phenomenon (fluorescence intensity decrease). The z-value is the interval of temperature required to change the D-value by a factor of 10. For isolated *GFPuv*, the higher z-value varied from 26.9°C

to 22.5°C, respectively, for the intervals between 45°C and 70°C and between 45°C and 75°C. The z-values dropped around 45% upon raising the heating temperature, from $z = 11.17^\circ\text{C}$ (interval of 70-95°C) to $z = 11.05^\circ\text{C}$ (interval of 75-95°C). Meanwhile, the process coefficient, Q_{10} ($Q_{10} = 10^{10/z}$), increased around three times, from $Q_{10} = 2.6$ to $Q_{10} = 8.0$, for these respective heating intervals. Therefore, the stability of isolated protein showed a greater dependence with temperatures higher than 70°C, when the D-value decreased eight times for every 10°C increase in the heating temperature. For temperatures $\leq 70^\circ\text{C}$, the protein was confirmed to exhibit greater stability.

For standard *GFPuv*, the z-value was about 12.9°C ($Q_{10} = 6.0$), between 75°C and 95°C and it was shown to be 33% (isolated *GFPuv* Q_{10} / standard *GFPuv*, $Q_{10} = 8.0/6.0$) more stable than the isolated protein, for the same temperature interval. The z-values for both standard and isolated *GFPuv* were comparable, with an average of 12.0°C. Through the evaluation of Q_{10} , the inactivation rate constant increased about 6 to 8 times in each additional 10°C interval. Thermal stability was shown to be similar for both standard and isolated *GFPuv*.

The rate of inactivation dependence upon treatment temperatures can be also described by energy of activation (E_a), by the Arrhenius equation. E_a indicates the stability of the system during heat treatment and relates the intrinsic energy of the system. Therefore, stable systems possess lower energy than unstable ones. E_a and z-value are related; inactivation reactions that exhibit large z-value and small E_a are less influenced by temperature; however, systems that have small z-value and high E_a have greater temperature susceptibility.

The energy of activation (E_a), in the interval of 75°C to 95°C, ranged from 53.0 kcal/mol to 45.5 kcal/mol (Figure 3), respectively, for both the isolated and standard *GFPuv*, with a variation of approximately 16%, which is not substantial. Therefore, both systems might have shown an equivalent thermal stability in this particular interval. For the interval between 60°C and 95°C, the E_a of 44.6 kcal/mol for the isolated *GFPuv* showed the same stability as standard *GFPuv* at 75-95°C and approximately 18% greater stability than the isolated *GFPuv* system at 75°C to 95°C. However, for isolated *GFPuv* at 45°C to 75°C, E_a dropped to 22.4 kcal/mol, about 42% less energy than the same system exposed at higher temperatures.

DISCUSSION

For sterilization processes which employ a chemical sterilant (such as ethylene oxide, hydrogen peroxide plasma, formaldehyde, unpublished data) with low temperature steam, *GFPuv* can be a potential BI at 45°C for 28h (1,680 min), at 60°C for 6h (360 min) and at 70°C for 3h (180 min), when the initial concentration of *GFPuv* is reduced by half.

To maintain the commercially desirable appearance and consistency of food products, blanching and pasteurization procedures are commonly used. Their efficiencies may be evaluated through the enzymatic activity of the most thermal resistant enzyme present in the product, such as pectinesterase, phenoxidase, peroxidase, catalase, and phosphatase. For peroxidase isoenzymes exposed to 75°C, Forsyth *et al.* (1999) obtained D-values between 0.01 min and 7.38 min for the native enzymes and from 0.096 min to 18.96 min for partially inactivated enzymes. These enzymes are employed as the BI to determine the efficacy of the blanching

procedure and to evaluate shelf life. For PME in citrus fruit juices exposed to the interval of 85-90°C, Eagerman and Rouse (1976) proposed z-values between 6.8°C and 17.6°C to extrapolate D-values at various temperatures. Heat induced chemical changes exhibit larger z-values relative to the killing of microorganisms. The typical z-value intervals are 4-7°C for some pathogenic bacteria, 10-20°C for the majority of enzymes related to product deterioration (Chen and Wu, 1998), 20-30°C for vitamins, and > 30°C for thermostable proteins. Due to the differences in z-values, it is apparent that short exposure at high temperatures can destroy pathogenic species but maintain the desirable characteristics of the product. Since the direct estimation of pathogen numbers by conventional microbial methods is time consuming, a simple test for the most resistant enzyme activity is appropriate on a routine basis. If enzyme activity is detected, it can be assumed that the heat treatment was inadequate (2-8). With the inactivation of *GFPuv* directly related to the time of exposure to moist-heat, *GFPuv* can be employed as BI in thermal processes (blanching, pasteurization, hygienization) at temperatures $\geq 75^{\circ}\text{C}$.

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Table 1. D-values of Isolated and Standard *GFPuv* obtained from heat treatments from

T = 45°C to 95°C with calculations of $\text{Log}_{10}C_f$ and R^2 .

<i>Isolated - GFPuv</i>				
Temperature (°C)	* $\text{Log}_{10}C_f$	R^2	D-value (hours)	D-value (minutes)
45	2.22 - 0.0003 t	0.98	55.55	3,333.00
60	2.24 - 0.0017 t	0.992	12.00	720.00
65	0.58 - 0.0018 t	0.996	10.41	625.00
70	0.50 - 0.0026 t	0.995	6.41	384.61
70	0.50 - 0.0027 t	0.998	6.16	370.00
75	0.46 - 0.0074 t	0.995	2.25	135.13
75	0.46 - 0.0077 t	0.995	2.16	129.87
80	0.45 - 0.0157 t	0.978		63.69
80	0.47 - 0.0183 t	0.984		54.64
85	0.64 - 0.0624 t	0.992		16.02
85	0.68 - 0.0634 t	0.991		15.77
90	0.79 - 0.114 t	0.998		8.77
90	0.89 - 0.1595 t	0.997		6.26
95	1.17 - 0.597 t	0.996		1.67
95	1.19 - 0.3992 t	0.994		2.50
<i>Standard GFPuv</i>				
Temperature (°C)	* $\text{Log}_{10}C_f$	R^2	D-value (hours)	D-value (minutes)
75	0.36 - 0.0063 t	0.98	2.65	158.73
80	0.37 - 0.0139 t	0.96	1.20	71.94
85	0.37 - 0.0333 t	0.99		30.03
90	0.56 - 0.0017 t	0.97		9.80
95	0.60 - 0.0034 t	0.98		4.90

* $\text{Log}_{10} C_f = \text{Log}_{10} C_o - (1/D)*t$, where C_o was the "initial concentration" and C_f was the "final concentration" of standard and isolated *GFPuv* ($\mu\text{g} / \text{mL}$), and t was the heating exposure time.

Figure 1. Decrease of *GFPuv* concentration (expressed in $\log_{10} \mu\text{g/mL}$) during exposure between 75°C and 95°C, for both standard *GFPuv* and isolated *GFPuv*.

Figure 2. Temperature dependence of D-values for isolated and standard *GFPuv* in Tris-HCl/EDTA, pH=8.0. Thermal parameters: z-value (inverse of the slope of the semi-logarithmic curve) and Q_{10} coefficient ($Q_{10} = 10^{10/z}$) calculated for both proteins

Figure 3. Temperature dependence of the reaction rate constant (k , min⁻¹), for isolated and standard *GFPuv* in Tris-HCl/EDTA, pH=8.0, by Arrhenius model. Energy of activation (E_a) calculated from the slope of the semi-logarithmic curve, for both proteins.

Anexo 09

“Thermal Stability of Recombinant Green Fluorescent Protein (*GFPuv*) at Various pH Conditions”

Trabalho apresentado na forma de poster no
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Colorado, Maio de 2003

Artigo a ser submetido a publicação

Este trabalho, como apresentado nesta dissertação, visou o estudo da termoestabilidade da proteína verde fluorescente recombinante *GFPuv* em diferentes temperaturas e valores de pH.

Conclusões

A estabilidade da proteína frente a diferentes temperaturas e valores de pH possibilita o seu uso como indicador biológico nos processos de esterilização.

Thermal Stability of Recombinant Green Fluorescent Protein (GFPuv) at Various pH Conditions

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ABSTRACT

The kinetic thermal stability parameters of the recombinant green fluorescent protein (*GFPuv*), expressed by transformed *Escherichia coli* DH5- α cells and isolated by TPP extraction with HIC were studied. The *GFPuv* at the concentration interval between 3.5 to 9.0 μg *GFPuv*/mL were exposed to various pH (4.91-9.03) and temperatures (75°C – 95°C) in the buffers: (i) 10 mM acetate (pH 5.0 to 7.0), (ii) 10 mM phosphate (pH 5.5 to 8.0) and (iii) 10 mM Tris-HCl (pH 7.0 to 9.0). The extent of protein denaturation by moist heat in the various buffers, as measured in the loss of fluorescence intensity, was expressed in decimal reduction time (D-value, at reference temperature), the interval of time required to reduce one decimal logarithm of the initial fluorescence intensity of *GFPuv*. For the pH between 7.0 and 8.0, the thermal stability of *GFPuv* was slightly greater in phosphate buffer than in Tris-HCl, even though Tris buffers are recommended for the *GFPuv* structure maintenance at pH $\geq$ 8.0. At 85°C, the mean D-value at pH 7.0 ranged from 8.33 min (acetate) to 13.88 min (phosphate) and, at pH > 8.50, ranged between 18.5 min and 27.7 min. The thermal stability for *GFPuv* in Tris-HCl (pH=8.0-8.9) was constant at 90°C and 95°C, the mean D-values were 7.93 min (pH=8.38 – 8.92) and 6.0 min (pH = 8.05 to 8.97), respectively. The thermal stability of *GFPuv* provides the basis for its potential utility as a fluorescent biological indicator to assay the efficacy of moist-heat treatments at temperatures lower than 100°C.

Key words: Green Fluorescent Protein (*GFPuv*); thermal stability; pH range; decimal reduction time (D-value).

INTRODUCTION

The biotechnological application of proteins to monitor the preservation of manufactured and processed products has expanded rapidly. Moist-heat treatments using steam at low temperatures ($T \leq 100^{\circ}\text{C}$; pasteurization, blanching, and sanitation) have been applied to food and pharmaceuticals to hinder microbial growth and inactivate endogenous enzymes to preserve and expand product shelf life. Moist-heat disinfection or hygienization is also utilized for the preservation of low-acid products ($\text{pH} > 4.5$) such as milk and other dairy products. The blanching efficiency is evaluated through the enzymatic activity of the most thermostable enzyme present in the product and this enzyme is usually selected and employed as the biological indicator (BI) to evaluate the immediate efficacy of thermal procedures, as well as to monitor the assurance of satisfactory preservation and shelf life of the product. These endogenous thermostable enzymes have been observed to exhibit greater thermal resistance than intact bacteria and yeast cells present in the product.

Makki and Durance (1) verified the thermal inactivation of lysozyme, an antimicrobial enzyme with considerable potential as a natural food preservative in vegetables, fresh fruits, seafoods and wine. The high thermostability of lysozyme makes it attractive to use in thermal processes like pasteurization, reducing nutritional and esthetic quality loss. Pasteurization associated with the blanching processing of fruit juices is necessary to prevent microbiological spoilage and to inactivate endogenous enzymes (2). Alkaline phosphatase, lactoperoxidase and other enzymes have been used for many years as an index of adequate pasteurization of milk (3). The pectinesterases are an example of endogenous enzymes that also have an important influence on the quality and stability of processed products (4). The dependence of the protein inactivation thermal process conditions (time and temperature) varies also with pH (5) and with the heating medium.

Due to the importance of validating the moist-heat treatments and related processes, it is necessary to develop a BI which can efficiently assess the success of the treatment. The use of a fluorescent marker designed for a quick and reliable assay, detectable by either microscopy, spectrofluorometry or hand-held UV lamp, is

examined in this work. The thermal stability of *GFPuv* provides the basis for its potential utility as a biological indicator, a fluorescent biological indicator to assay the efficacy of moist heat treatments ($T < 100^{\circ}\text{C}$).

GFPuv is a compact, globular acidic protein (pI 4.6 to 5.4) with one fluorophore consisting of a cyclic tripeptide in the primary protein sequence, a chain of 238 amino acids. It is resistant to heat ($T > 70^{\circ}\text{C}$), alkaline pH (between pH 5.5 and 12.0; the optimum pH = 8.0), and some detergents, inorganic and organic reagents, and solvents.

The term “thermal stability” refers to the resistance of a protein to adverse intrinsic and extrinsic environmental influences, that is, the thermal characteristic of the protein to remain steady against the denaturation of its molecular integrity and inactivation of its biological activity on facing high temperatures or other deleterious agents (6). One of the most important indices to measure protein stability is the decimal reduction time, D-value, the time required to reduce 90% of the initial protein concentration exposed to the reference temperature. The D-value was used as the main kinetic parameter to compare the influence of environmental conditions on the thermal behavior of *GFPuv*.

The purpose of this work is to determine the thermal stability of the TPP-extracted recombinant protein, *GFPuv*, in different buffers, as well as to verify its utility as a quick, accurate and economical biological indicator for moist heat-treatments ($T < 100^{\circ}\text{C}$) in different pH conditions.

MATERIAL AND METHODS

Transformation and Expression. *Escherichia coli* DH5- α cells (7) transformed (pGFPuv, Clontech, CA, USA) by the standard calcium chloride method (8,9) to express the recombinant green fluorescent protein (*GFPuv*, excitation/emission maxima at 394nm/509nm) were grown (37°C / 100 rpm/ 24h) in Luria Bertani broth (LB, USB, Ohio, USA) supplemented with 100 $\mu\text{g/mL}$ ampicillin (amp, Boehringer, Mannheim, Germany). An aliquot of this culture was transferred onto the surface of LB-amp agar with isopropyl- β -D-thiogalactopyranoside (IPTG) added to final concentration of 0.5 mM (w/v) and

incubated at 37°C. After 24h, the agar surface was illuminated with a hand-held UV lamp at 395 nm (Model UVL 4, UVP, CA, USA) and isolated brightly fluorescent colonies were picked and transferred into 25 mL LB-amp broth (pre-inoculum), and incubated (100 rpm/37°C) up to an $OD_{660} = 0.01$ (10^4 CFU/mL; spectrophotometer, Beckman DU-600, CA, USA). A 1 mL aliquot of this pre-inoculum was transferred into 25 mL LB-amp broth, and the 250 mL flasks were shaken (100 rpm/ 37°C) until the broth cultures attained an $OD_{660} = 0.8$ (10^7 CFU/mL). IPTG was then added to a final concentration of 0.5 mM. After another 21h (100 rpm/ 37°C), the expression of *GFPuv* by induced cells was detected by illuminating the broth at 395 nm and visually inspecting for intense green fluorescence. Cells were harvested by centrifugation (6.000xg/ 30 min/ 4°C). The supernatant was decanted and the cell pellet was resuspended in 4 mL of chilled (4°C) extraction buffer, ("XE": 25.0 mM Tris-HCl, pH 8.0, 1.0 mM β -mercaptoethanol, " β -ME", Pharmacia Biotech, Sweden; 0.1 mM phenylmethylsulphonylfluoride, "PMSF", USB, Switzerland) prior to subjecting the cells to the three-phase partitioning extraction (TPP) method.

Three-Phase Partitioning Extraction (TPP) Method. (10,11) and HIC purification. In a 2 mL microcentrifuge tube, to each aliquot of 450 μ L of resuspended cells, 300 μ L of 4 M $(NH_4)_2SO_4$ and 750 μ L of *t*-butanol were added. The mixture was stirred for 2 min at room temperature, centrifuged and the three phases formed were collected separately. The *t*-butanol upper phase and the white interfacial precipitate were removed and discarded. A second aliquot of 750 μ L of *t*-butanol was mixed into the lower aqueous phase. The mixture was allowed to settle to visible phase separation and centrifuged. At the separation of the three phases, the upper phase was discarded. The interfacial green phase was collected and dissolved in 500 μ L XE. While the lower phase was still fluorescent, it was subjected to repeated TPP extraction. A 1.0 mL aliquot of protein extract was mixed with 1.0 mL 4 M $(NH_4)_2SO_4$ and this mixture was transferred to the top of a methyl support hydrophobic interaction column ("HIC"), fast flow, for final purification. The HIC was previously equilibrated with 2 M $(NH_4)_2SO_4$. The loaded column was first washed with 250 μ L 1.3 M $(NH_4)_2SO_4$ to elute proteins that bind with low affinity to

the methylated resin of the column packing. *GFPuv* was eluted with 1.0 mL of buffer solution (10 mM Tris-HCl, 10 mM EDTA, pH 8.0), and stored at 4°C.

The fluorescence intensity (± 0.005) of *GFPuv* was measured in the eluted samples in a spectrofluorometer (excitation, 394nm/emission, 509nm filters, RF 5301 PC, Shimadzu Corporation, Kyoto, Japan). Purified recombinant *GFPuv* ("standard *GFPuv*"; Clontech, USA) was used to generate a standard curve to determine TPP-extracted *GFPuv* concentration and provide an experimental comparison for thermal stability. The fluorescence intensity of the TPP-extracted samples were compared with the standard calibration curve (1): fluorescence intensity (I) = $-33.078 + 38.943 \times (GFPuv \text{ } \mu\text{g/mL})$; ($R^2 = 0.997$). To determine the concentration of the TPP-extracted *GFPuv*, fluorescence intensity was correlated to the concentration of native *GFPuv*, since denatured *GFPuv* cannot fluoresce, and the loss in fluorescence intensity was a measure of denatured *GFPuv* concentration.

Moist-heat treatment of extracted GFPuv under different pH conditions.

Thermal Stability of GFPuv at different pH values. Samples of TPP-extracted/purified *GFPuv* were exposed (24 h/ 4°C) to different pH values (5.0 ± 0.2 to 9.0 ± 0.2) in buffer solutions: (i) 10 mM acetate (pH 5.0 to 7.0), (ii) 10 mM phosphate (pH 5.5 to 8.0) and (iii) 10 mM Tris-HCl (pH 7.0 to 9.0) before heating. To each 3900 μL buffer solution, 100 μL TPP-extracted sample (at initial concentration around 420 $\mu\text{g GFPuv/mL}$) was mixed. The mixture (4000 μL) was stored at 4°C over night, and centrifuged (6000 g/ 30 min). Precipitation was not observed. The pH values of the buffer solutions were also measured and correlated with the correspondent $\mu\text{g GFPuv/mL}$ by the standard calibration curve (1). The pHmeter AR-20 (Fisher Scientific, USA) was previously adjusted with standard buffers (Synth, Brazil) of known pH values: 4.0, 7.0 and 9.0. One mL sample was transferred to a quartz cuvette [(3 mm x 10 mm) light path length x 45 mm height] and sealed with a plastic cover. Each cuvette was inserted into the adapter assembly and adjusted in the cell holder. The cell holder was connected to a constant-temperature bath and the circulating water stabilized at the assay temperature. A constant-temperature water circulating device (Thermo-bath TB-85 P/N 200-65022, Shimadzu, Kyoto, Japan), was used to heat and control the temperature of the cell

holder. The temperature of the waterbath was maintained within $\pm 0.05^\circ\text{C}$, and provided constant-temperature, by continuous circulation of water from the waterbath, to the cell holder and the sample in the cuvette via a circulation pump. From the moment the sample-filled cuvette was placed in cell holder and the heat-treatment was initiated, a fluorescence reading was taken at defined exposure intervals at different temperatures (75°C , 80°C , 85°C , 90°C and 95°C). The variation in fluorescence intensity (± 0.005) was measured by the spectrofluorometer (excitation/emission maxima at 394/509 nm) every 20s and converted to native *GFPuv* concentrations by the standard calibration curve (1).

Analysis of the Thermal Kinetic Parameters. The *GFPuv* inactivation curves were considered first order models represented by $\text{Log}_{10} C_f = \text{Log}_{10} C_o - (1/D) \cdot t = \text{Log}_{10} C_o - (k/2.303) \cdot t$, where C_o was the "initial concentration" of native *GFPuv* ($\mu\text{g}/\text{mL}$) and C_f was the "final concentration" of native *GFPuv* ($\mu\text{g}/\text{mL}$), after the exposure to the heating time (t , min), D -value, the decimal reduction time (D , h or min) and the inactivation rate constant (k , h^{-1} , min^{-1}).

The decimal reduction time, D-value, the interval of time required to reduce one decimal logarithm of the initial fluorescence intensity of *GFPuv* at reference temperature, was determined from the negative reciprocal of the slopes of the regression lines, using the linear portions of the inactivation curves ($\log_{10} \mu\text{g GFPuv}/\text{mL}$ versus time of exposure at a constant temperature). The final heated *GFPuv* concentration considered to estimate D -values ranged from 1.00 to 1.55 $\mu\text{g GFPuv}/\text{mL}$. *The temperature coefficient, Q_{10}* , was calculated to evaluate the dependence of the D -value on an increase of 10°C in the heating temperature (T , $^\circ\text{C}$), by the following relation: $Q_{10} = [D_T/D_{(T+10)}]$. *The z-value ($^\circ\text{C}$)*, expressed as the number of degrees ($^\circ\text{C}$) required for one Log_{10} change in the D -value ($D=2.303/k$), was determined by plotting the relation between Log_{10} D -value and corresponding temperatures. The z -value may be related to the coefficient Q_{10} of the process by: $Q_{10} = (10^{10/z})$. *Activation energy (E_a , kcal/mol)* was estimated by using the Arrhenius equation, which was applied to evaluate the dependence of the inactivation rate constant, k , on the heating temperature: $\text{Log}_{10} k_1 = \text{Log}_{10} k_2 - \{(E_a/2.303 \cdot R)[(1/T_1) -$

$(1/T_2)]\}$, where T_1 and T_2 are the heating temperatures in degrees Kelvin (K), and R is the universal gas constant ($1.987 \text{ calories} \cdot \text{mol}^{-1} \text{ K}^{-1}$).

RESULTS

Thermal treatments. Recombinant green fluorescent protein, *GFPuv*, expressed by *E. coli*, isolated by the TPP-extraction method and eluted from a methyl HIC column (“extracted *GFPuv*”), was diluted in buffers and stored at 4°C for stabilizing the solution pH values before heating. After 24h/ 4°C , the concentration of *GFPuv* varied in accordance with buffer solution and respective pH-values. In acetate buffer, the lower *GFPuv* concentration ranged from 1.34 to $5.87 \mu\text{g GFPuv/mL}$ at pH between 4.91 and 5.72; and the higher *GFPuv* concentration ranged from 6.20 to $9.20 \mu\text{g GFPuv/mL}$ at pH between 6.13 and 7.03. In phosphate buffer, the lower *GFPuv* concentration ranged from 4.89 to $5.96 \mu\text{g GFPuv/mL}$ at pH between 5.67 and 5.97; and the higher *GFPuv* concentration ranged from 5.31 to $9.08 \mu\text{g GFPuv/mL}$ at pH between 6.19 and 8.00. In Tris-HCl buffer, the *GFPuv* concentration ranged from 5.52 to $9.33 \mu\text{g GFPuv/mL}$ at pH between 6.67 and 9.03. The greater loss of fluorescence intensity for *GFPuv* in acetate buffer was around 74% at pH 4.92, lowering the loss to 48.90% at pH = 5.32, decreasing to 10% of loss at pH = 5.66 and to 2.0% at pH > 6.0. In phosphate buffer and Tris-HCl the maximum losses of fluorescence intensity occurred around $15.0 \pm 5.0\%$. The stability of fluorescence intensity was verified for *GFPuv* in phosphate and Tris-HCl buffers at pH ≥ 6.40 . The effect of pH on the standard and TPP-extracted/purified *GFPuv* was previously studied and confirmed that a quite good stable fluorescence intensity for *GFPuv* was characterized in the pH range from 6.0 to 9.8, declining abruptly for pH between 5.5 and 4.5; being the fluorescence intensity minimum at pH values lower than pH 4.5 (9). Bokman & Ward (12) verified that native *GFPuv* maintains stable fluorescence in the pH range from 5.5 to 12.0; however, fluorescence intensity decreases between pH 5.5 and 4.4, and drops sharply above pH 12.0.

The interval of initial concentration (C_0) of *GFPuv* being subjected to continuous heating at set waterbath temperatures in buffer solutions varied from 3.5 $\mu\text{g GFPuv/mL}$ to 9.0 $\mu\text{g GFPuv/mL}$.

The variation in fluorescence intensity was measured by the spectrofluorometer and converted to the concentration of native *GFPuv*. After heating and repeated exposure to light during the assay, the *GFPuv* samples were stored for 24h at 4°C and fluorescence intensity was again measured and compared to final heat-treatment intensity. No increase of fluorescent intensity was observed that may be related to the recovery of the native conformation of the protein. Post-heating, the pH did not change and the samples remained clear, without any protein precipitating out of solution.

Decimal reduction time (D-value). The denaturation of *GFPuv* in buffer solutions at various pH values was measured by the loss of fluorescence intensity and expressed in the decimal logarithm of the decrease in native *GFPuv* concentration versus the time of exposure at constant temperature (Table 1, Figure 1). To estimate D-values at constant heating temperatures and pH value, the interval of *GFPuv* concentrations considered was: (i) between 3.5 $\mu\text{g/mL}$ (acetate, pH 5.18) and 9.0 $\mu\text{g/mL}$ (Tris, pH > 8.0, for initial concentration (C_0); and ranged from 1.00 $\mu\text{g/mL}$ to 1.55 $\mu\text{g/mL}$, for final concentration (C_f), which corresponded to the linear portion of the inactivation curves ($\log_{10} \mu\text{g GFPuv/mL}$ versus time of exposure at a constant temperature and pH value, Figure 1).

At 90°C and 95°C, a slight drop in fluorescence intensity was observed (Figure 1) without interfering in the determination of D-value. For lower temperatures, the loss of fluorescence intensity decreased gradually. For the same temperature, the thermal stability of *GFPuv* was observed to gradually increase proportionally with pH, and was shown to be dependent upon the buffer used.

At 75°C, the mean D-values for *GFPuv* attained their maximum in acetate at pH 6.6-7.0 ($D = 41.60$ min), doubled in phosphate at pH 7.6-8.0 ($D = 104.16$ min) and with a three-fold increase in Tris-HCl at pH 8.1-8.5 ($D = 125.00$ min). The phosphate buffer system was shown to provide greater thermal stability to the protein in the interval of pH between 6.6 and 8.0. The D-values for *GFPuv* were not reproducible at pH > 8.5 in Tris-HCl buffer.

However, at pH 6.1-6.5, the D-values for *GFPuv* in acetate were double those for *GFPuv* in phosphate, and at 6.6-7.0 the thermal behavior of *GFPuv* was equivalent for both buffers, although the greater variation of D-values was seen in phosphate buffer.

At 80°C and 85°C, respectively, the D-values for *GFPuv* in acetate (D=20.8 min and 8.33 min) and in Tris-HCl (D=23.8 min and 7.24 min) at pH 7.1-7.5 were close and half the D-values (D=55.55 min and 13.88 min) observed in phosphate. Therefore, the phosphate buffer provided the optimal buffering system to maintain the thermal *GFPuv* stability in an important range of pH 6.0 to 8.0. At pH > 8.0, the Tris-HCl solutions were confirmed to maintain the thermal *GFPuv* stability.

At $\geq 85^\circ\text{C}$, D-values dropped at least twice for every buffer system. The D-values were similar for *GFPuv* in phosphate and acetate buffers at 90-95°C, pH 5.5-7.0. The thermal stability of *GFPuv* was the highest in the phosphate buffer at pH 7.6-8.0 and Tris-HCl at pH > 8.0. At pH 7.6-8.0 in phosphate and pH > 8.0 in Tris-HCl buffer, the highest thermal stability of *GFPuv* was shown to be equivalent. Although, Tris-HCl buffer at pH 8.0 is used to store and maintain *GFPuv*, phosphate buffer provided equivalent thermal protein fluorescence stability.

D-VALUES' CORRELATION WITH pH AND BUFFER SYSTEMS

Stability of GFPuv at different pH values:

According to Vessoni Penna (9), a good stability of the *GFPuv* molecule started to be observed at pH around 5.0 – 5.2, corresponding to our findings on the minimum thermal *GFPuv* stability that was shown at the pH 4.91, referent to acetate buffer and treatment at 85°C, very close to the isoelectric point (pI) of the protein. Ward (13) verified that the pI for the native GFP was between pH 4.7 and 5.1, corresponding with our finding on *GFPuv* extraction efficiency.

The thermal stable fluorescence intensity of *GFPuv* was shown in the pH range from 6.0 to 8.5. However, at pH 8.5-9.0, the fluorescence intensity was variable at 75°C in Tris-HCl and was kept constant for the higher temperatures, considering the same buffer system. Therefore, the range of pH for different buffers

and treatments was linear correlated to D-values, which proportionally to the increase of pH followed higher thermal *GFPuv* stability.

The linear correlation between extreme pH intervals and D-values was shown to be dependent upon buffer systems and heating temperatures, as shown in Table 2, from: (i) pH 4.91 (85°C) to 7.03 (85°C) for acetate; (ii) pH 5.63 (85°C) to 7.91 (80°C) for phosphate and (iii) pH 6.91 (75°C) to 9.03 (90°C) for Tris-HCl solutions.

Kinetic parameters z-value, Q_{10} coefficients and activation energy (E_a)

The kinetic parameters z-value and the related Q_{10} coefficient ($Q_{10} = 10^{10/z}$) reflect the temperature dependence of the denaturation phenomenon (fluorescence intensity decrease). The z-value is the interval of temperature required to change the D-value by a factor of 10.

For every temperature and buffer, the correspondent D-values at pH between 6.0 and 8.5 were calculated from the set equations shown in Table 2. The respective z-values (°C), were determined from the negative reciprocal of the slopes of the regression lines of the relations between $\log_{10}$ D-value and corresponding temperatures. The Q_{10} coefficients were estimated through the relation with z-values ($Q_{10}=10^{(10/z)}$). Those parameters are shown in Table 3.

The thermal stability of *GFPuv* was closely maintained at pH > 6.0 in the buffers: (i) acetate at pH 6.5 ($z = 23.42^\circ\text{C}$) and pH 7.0 ($z = 20.45^\circ\text{C}$); (ii) phosphate at pH 7.0 ($z = 16.64^\circ\text{C}$), pH 7.5 ($z = 14.01^\circ\text{C}$) and pH 8.0 ($z = 13.75^\circ\text{C}$); (iii) Tris-HCl at pH 7.5 ($z = 15.41^\circ\text{C}$), pH 8.0 ($z = 15.6^\circ\text{C}$) and pH 8.5 ($z = 14.95^\circ\text{C}$). At pH 6.0, the z-value of 30.96°C for *GFPuv* in acetate solution led to the highest thermal stability of the protein for the temperature interval between 75-95°C. The z-values dropped around 25% upon pH 7.0 and 7.5, from $z = 23.42^\circ\text{C}$ (acetate buffer) and 22.42°C (phosphate buffer) to $z = 20.45^\circ\text{C}$ (acetate) and 20.79°C (Tris-HCl), respectively. At pH = 8.0, the z-values dropped by half, between 13.75°C and 15.6°C considering phosphate and Tris-HCl buffers, respectively.

Meanwhile, the process coefficient, Q_{10} ($Q_{10} = 10^{10/z}$) around 3.0, suggested that acetate and Tris-HCl buffers at pH 7.0 provided the similar stabilization for the protein's thermal characteristics greater than phosphate ($Q_{10} = 3.99$), which system exposed *GFPuv* to more dependence on the temperature change. At pH 8.0, the

protein in Tris buffer solution was confirmed to exhibit greater stability, when the D-value decreased four times for every 10°C increase in the heating temperature. At the same pH 8.0, the inactivation rate constant increased 5.34-fold for the protein in phosphate system, which provided the greatest dependence of *GFPuv* to the treatment temperatures.

The rate of inactivation dependent upon treatment temperatures can be also described either by energy of activation (E_a), by the Arrhenius equation. E_a indicates the stability of the system during heat treatment. E_a relates the intrinsic energy of the system. Therefore, stable systems possess lower energy than unstable ones. E_a and z-value are related; inactivation reactions that exhibit large z-value and small E_a are less influenced by temperature; however, systems that have small z-value and high E_a have greater temperature susceptibility, as shown in Table 3.

The *GFPuv* stability was greater in acetate at pH 6.0 ($E_a = 19.27$ kcal/mol); equivalent ($E_a \sim 26.50$ kcal/mol) at pH 6.5 in acetate and phosphate; and at pH 7.0 in acetate and Tris-HCl. For the same buffers, upon the increase in pH to 7.5-8.5, the thermal vulnerability of *GFPuv* incremented 30% the E_a range from 38.15 to 39.9 kcal/mol. Otherwise, the solutions of *GFPuv* in phosphate at pH 7.5 and 8.0 ($E_a = 42.63$ and 42.67 kcal/mol) established the lowest thermal stability for *GFPuv*.

For pH > 7.0, the $Q_{10} > 5.0$ coefficient showed D-values 13% more vulnerable for *GFPuv* in phosphate than in Tris-HCl and acetate buffers, confirming its role in the improved maintenance of *GFPuv* conformation and fluorophore emission output.

The thermal stability of *GFPuv* was similar for the systems: (i) acetate solutions at pH interval of 6.5- 7.0, and respective z-values of 23.42°C and 20.45°C; (ii) phosphate solutions at between the pH of 7.0- 8.0, and respective z-values of 16.64°C, 14.01°C and 13.75°C; (iii) Tris-HCl at the pH interval of 7.5 - 8.5 and respective z-values of 15.41°C, 15.6°C and 14.95°C.

The thermal stability of *GFPuv* was: (i) higher when heated in acetate solution pH 6,0 ($E_a = 19.27$ kcal/mol), and (ii) equivalent for the systems provided by acetate solutions at the pH interval of pH 6.5- 7.0 ($E_a = 25.15$ and 28.76

kcal/mol), phosphate at pH 6.5 ($E_a = 26.18$ kcal/mol) and Tris-HCl at pH 7.0 ($E_a = 28.19$ kcal/mol).

The data confirmed the previously (14) thermal stability of standard *GFPuv* in Tris-HCl buffer at pH 8.5, when the z -value was about 12.9°C ($Q_{10} = 6.0$, $E_a = 45.54$ kcal/mol), comparable with an average of $z = 13.75^\circ\text{C}$ ($Q_{10} = 5.34$) observed for the extracted protein heated in phosphate buffer at pH 8.0, and the system corresponded to an average $E_a = 42.67$ kcal/mol, for the same interval of temperature, between 75°C and 95°C .

DISCUSSION

Laratta (15) studied five tomato varieties and all varieties showed the same thermal performance: the thermal pectin methyl esterase ("PME") resistance was higher at higher pH. All PME varieties showed a typical biphasic performance, characterized by a lower thermal resistance at temperatures lower than 74°C and a higher thermal resistance above that temperature. The temperature range between 90 and 115°C is generally employed in the food industry to microbiologically and enzymatically stabilize these products. The z -values for the five varieties of tomatoes varied from 18.9°C to 36°C related to a pH range from 3.9 to 4.9, which was responsible, in my point of view, for a low interval of D -values, between 0.16–0.18 min. The variation on the thermal stability of *GFPuv*, verified in the present work, related to the three buffers, was similar considering the z -value range from 13.75°C in phosphate (pH 8.0) to 30.96°C for acetate (pH 6.0) solutions, corresponding to an interval of D -value between 4.06 min (pH = 5.6–6.0) and 6.08 min (pH = 7.6–8.0) at 95°C .

Forsyth (16) studied the thermal performance of peroxidase at 75°C , and obtained a D -value range from 0.01min to 7.38 for the native enzyme and between 0.096 min and 18.96 min for the partially inactivated enzyme.

Griffitts (3) studied the thermal stability of the naturally occurring enzymes in milk in the temperatures between 65 – 80°C , in order to choose an enzymatic index of adequate pasteurization of milk. For the acid phosphatase, the D -values of 7.38

min at 75°C and 7.87 min at 80°C corresponded to a z-value of 6.6°C; for the inactivation of lactoperoxidase, D-values of 0.80 min at 75°C and 0.075 min at 80°C corresponded to a z-value of 5.4°C; for amylase (saccharifying activity), D-values of 0.85 min at 75°C and 0.45 min at 80°C corresponding a z-value of 16.2°C. The naturally occurring enzymes in milk showed lower D-values than those found for *GFPuv* in the three buffers. However, z-value of 16.64°C characterized for *GFPuv* in phosphate buffer solutions at pH 7.0 was similar to that obtained for amylase in milk.

The thermal kinetics for lysozyme in beer (1) was characterized for systems containing 15% of glucose at pH 5.20 and 7.20, corresponding respectively to z-values of 13.8°C and 14.1°C, to E_a values of 42.0 kcal/mol and 41.1 kcal/mol. While, for systems containing 1.0 M NaCl at pH 7.20, the obtained parameters were z-value of 13.9°C and E_a value of 41.8 kcal/mol. For both systems, the kinetic parameters were similar to those verified for *GFPuv* in phosphate solutions at pH 7.5 and 8.0.

Thermal inactivation of *B. cereus* spores were confirmed to be affected by the solutes used to control water activity (A_w) of the heating medium. For the spores of *B. cereus* ATCC 7004 subjected to heating interval between 92°C and 106°C, Mazas et al. (17) determined the following parameters: (i) z-value of 8.3°C for spores in water ($A_w = 1.0$) at pH 7.60, as a medium control; (ii) z-value of 10.3°C, for spores in 5.6 M NaCl solution, at pH 8.35 and $A_w = 0.75$. For both extreme systems, the spores of *B. cereus* exhibited lower thermal stability than even to the system of *GFPuv* in phosphate solution (z minimum of 13.75°C). Among the environmental conditions that affect spore thermoresistance, (18) stated that pH value is a major intrinsic factor in microbial destruction, as well as in the recovery medium of the injured spores.

Since the direct estimation of microbial numbers by conventional culturing methods is time consuming and well affected by the environment conditions (18), a simple test for the most resistant enzyme activity is appropriate on a routine basis. If enzyme activity is detected, it can be assumed that the heat treatment was inadequate (4,15). Appropriately, *GFPuv* can be employed as bioindicator in thermal processes (blanching, pasteurization, hygienization) at temperatures $\geq 75^\circ\text{C}$ for products at pH

> 5.5, with the inactivation of *GFPuv* shown to be directly related to the time of exposure to moist-heat and pH.

CONCLUSIONS

The thermal stability of *GFPuv* provides the basis for its potential utility as a fluorescent biological indicator to assay the efficacy of processes at temperatures lower than 100°C, in an interval of pH between 5.5 and 8.5, and dissolved in various buffers.

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Figure 1. Inactivation curves for the recombinant green fluorescent protein *GFPuv* in: (i) acetate buffer at pH 6.0; phosphate buffer at temperature 85°C in different pH values; (iii) Tris-HCl at pH 8.5. The Decimal reduction times were determined from the reciprocal of the slope of the linear portion of the *GFPuv* inactivation curves, considered first order models.

(i) Inactivation curves for *GFPuv* in acetate buffer at pH 6.0.

(ii) Inactivation curves for *GFPuv* in phosphate buffer at temperature 85°C in different pH values.

(iii) Inactivation curves for *GFPuv* in Tris-HCl at pH 8.5.

Table 1. D-values at Reference Temperatures and pH Intervals.

Temperature (°C)	Buffer Solution (10mM)	¹ D-values (min)								
		pH values								
		5.0 - 5.5	5.6 - 6.0	6.1 - 6.5	6.6 - 7.0	7.1 - 7.5	7.6 - 8.0	8.1 - 8.5	8.6 - 9.0	
75	Acetate	7.60±2.39	7.93± 2.40	21.40±7.13	41.60±0.48					
	Phosphate		12.89±1.12	11.54±3.94	58.33±28.29	76.38±13.61	104.16±40.83			
	Tris-HCl				25.7±22.04	30.52±3.19	69.44±15.72	125.00±47.15	52.08±35.36	
80	Acetate	5.74±1.12	7.24±1.20	10.14±1.50	17.95±3.23	20.8±2.20				
	Phosphate			9.72±0.55	23.80± 0.62	55.55± 0.73	84.15±0.96			
	Tris-HCl					20.83± 0.75		55.55± 0.64	55.55± 0.42	
85	Acetate	3.71±0.80	4.76±	5.55±	7.09±0.17	8.33±				
	Phosphate		4.38±0.36	6.40±0.96	13.88±	13.88±	17.67±1.65			
	Tris-HCl					7.24± 1.20	10.57±1.50	18.51± 1.10	27.75±0.06	
90	Acetate	3.26±0.80	3.96± 0.45	4.62± 0.10	5.13±0.08	5.05±0.13				
	Phosphate			4.38±0.09	5.74± 0.07	6.66± 0.05	6.94± 0.09			
	Tris-HCl					3.56±0.73	4.91±0.16	6.25±1.29	5.5±2.62	
95	Acetate	3.74±0.58	4.06±0.47	4.38±0.32	4.70±0.23	4.9± 0.26				
	Phosphate		3.78±0.05	4.44±0.07	5.96±0.24	5.95±0.33	6.08±0.38	5.42±0.06		
	Tris-HCl			2.92±0.72		2.46±0.69	3.35±0.79	5.17±2.51	5.48±2.05	

n = 10 observations

p < 0.05

C.I. = confidence interval

¹ (D ± CI) min

Table 2. Equations to determine D-values for *GFPuv* in various buffers exposed to different conditions of temperature and pH.

Buffer Solution	Temp. (°C)	pH Range	Equation D - value	R ²
Acetate	75	5.5 - 7.01	$= - 138.09 + 25.677 \cdot \text{pH}$	0.97
	80	5.18 - 7.01	$= - 41.644 + 8.8106 \cdot \text{pH}$	0.94
	85	4.91 - 7.03	$= - 12.1 + 2.8747 \cdot \text{pH}$	0.94
	90	5.19 - 7.01	$= - 1.9854 + 1.0318 \cdot \text{pH}$	0.97
	95	5.46 - 7.01	$= - 0.9143 + 0.8296 \cdot \text{pH}$	0.91
Phosphate	75	6.19 - 7.20	$= - 392.14 + 64.603 \cdot \text{pH}$	0.92
	80	5.97 - 7.91	$= - 271.76 + 45.632 \cdot \text{pH}$	0.96
	85	5.63 - 7.84	$= - 35.676 + 6.8552 \cdot \text{pH}$	0.94
	90	6.03 - 7.80	$= - 5.6922 + 1.6322 \cdot \text{pH}$	0.93
	95	5.89 - 7.82	$= - 3.6824 + 1.3041 \cdot \text{pH}$	0.97
Tris-HCl	75	6.91 - 8.50	$= - 636.99 + 92.299 \cdot \text{pH}$	0.89
	80	7.50 - 8.84	$= - 182.68 + 27.51 \cdot \text{pH}$	0.91
	85	7.41 - 8.90	$= - 91.089 + 13.097 \cdot \text{pH}$	0.94
	90	7.27 - 9.03	$= - 14.998 + 2.5629 \cdot \text{pH}$	0.77
	95	7.08 - 8.92	$= - 30.48 + 4.5551 \cdot \text{pH}$	0.98

Table 3. Kinetic parameters of thermal stability for *GFPuv*: z-values (°C), Q_{10} coefficients and activation energy (Ea, kcal/mol)

pH	Acetate			Phosphate			Tris-HCl		
	Z-value (°C)	Q_{10}	Ea (kcal/mol)	Z-value (°C)	Q_{10}	Ea (kcal/mol)	Z-value (°C)	Q_{10}	Ea (kcal/mol)
6.0	30.96	2.10	19.27						
6.5	23.42	2.67	25.15	22.42	2.79	26.18			
7.0	20.45	3.08	28.76	16.64	3.99	35.28	20.79	3.03	28.19
7.5				14.01	5.17	42.63	15.41	4.46	38.15
8.0				13.75	5.34	42.67	15.6	4.38	38.2
8.5							14.95	4.67	39.9