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APLICAÇÃO DE PROTEASES ALCALINAS DAS VÍSCERAS DO TAMBAQUI (*Colossoma macropomum)* E DA CARPA *(Cyprinus carpio)* COMO ADITIVO DE DETERGENTES EM PÓ

TALITA DA SILVA ESPÓSITO

RECIFE
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Dissertação apresentada ao **Programa de Pós-Graduação em Recursos Pesqueiros e Aqüicultura** da Universidade Federal Rural de Pernambuco, como parte dos requisitos necessários para a obtenção do grau de **Mestre em Recursos Pesqueiros e Aqüicultura.**

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Recife, 06 de fevereiro de 2006

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Aos meus pais, Ivaldino e Aparecida Espósito,
que por tantas vezes abdicaram dos seus sonhos
para que eu pudesse realizar os meus

“Não haveria criatividade sem a curiosidade
que nos move e que nos põe pacientemente
impacientes diante do mundo que não fizemos,
acrescentando a ele algo que fazemos”

Paulo Freire

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LISTA DE ABREVIATURAS

IUBMB - International Union of Biochemistry and Molecular Biology

pH - potencial hidrogeniônico

EC - Enzyme Commission

kDa - quilo Daltons

SDS - sódio dodecil sulfato

SDS-PAGE - eletroforese em gel de poliacrilamida utilizando SDS

PMSF - fenil-metil-sulfonil-fluoreto

TPCK - tosil-amido-2-feniletil clorometil cetona

TLCK - tosil-lisina clorometil cetona

BAPNA - benzoil arginina ρ-nitroanilida

SUCPHEPNAN - succinil fenilalanina ρ -nitroanilida

RESUMO

As proteases alcalinas são as enzimas de maior importância comercial devido a sua vasta aplicabilidade, sendo empregadas principalmente nas indústrias de detergentes e de alimentos. Atualmente, a maior parte das proteases utilizadas no mercado é produzida por bactérias do gênero *Bacillus*, no entanto, elas podem ser amplamente encontradas em um dos principais resíduos da indústria pesqueira, as vísceras. Portanto, uma forma de otimizar o aproveitamento do pescado e, conseqüentemente, reduzir o desperdício é reaproveitar esses subprodutos. Neste trabalho testou-se a aplicabilidade das proteases de vísceras de peixes como aditivo de detergentes em pó comerciais. Para extração das enzimas foram utilizadas vísceras de *Colossoma macropomum* e de *Cyprinus carpio*, principal peixe nativo e segundo peixe exótico da aqüicultura continental nacional, respectivamente. A partir deste material obteve-se o extrato bruto, que passou por uma semi-purificação fracional com etanol. As frações obtidas deste procedimento tiveram sua atividade enzimática e quantidade de proteínas determinadas para escolha da fração a ser trabalhada. A fração saturada com 30-70% de etanol apresentou maior atividade específica tanto no tambaqui quanto na carpa, com rendimentos de 74,9% e 142,4%, respectivamente. Nesta fração verificou-se em que temperatura e pH as proteases apresentavam maior atividade, além da sua estabilidade em relação a esses parâmetros. Para testar a compatibilidade com detergentes comerciais, foram utilizados quatro detergentes comerciais, cinco agentes surfactantes e peróxido de hidrogênio em diferentes concentrações. Os resultados obtidos sugerem que as proteases alcalinas encontradas nas vísceras de tambaqui apresentam características ideais para utilização na indústria de detergentes em pó, como: retenção de até 73% da sua atividade na presença de Surf® e de 63% na presença de 5% de H_2O_2 após 1 hora de incubação a 40ºC. Além disso, a atividade da enzima foi estimulada na presença de surfactantes não-iônicos (Tween 20 e Tween 80) e iônicos (Saponin e Colato de sódio). As proteases obtidas a partir do intestino da carpa, no entanto, só foram estáveis na presença dos diferentes surfactantes e a 5% de H_2O_2, perdendo estabilidade quando adicionadas aos detergentes comerciais.

ABSTRACT

Alkaline proteases are commercially important group of enzymes and have a large variety of applications, mainly in the detergent and food industries. The proteases currently uses in the market are serine proteases produced by *Bacillus* strains, principally. Fish viscera have wide biotechnological potential as a source of digestive proteases. Biotechnology provides a means for transforming such materials usually discarded into valuable produts such as enzymes. The objective of this research was to test if alkaline proteases from fish viscera could be used as an additive in commercially available detergent formulations. Viscera from *Colossoma macropomum*, the most important native fish for Brazilian aquaculture, and *Cyprinus carpio*, the second exotic fish in importance for Brazilian aquaculture, were extracted and used as a source of enzyme for this research. The crude extract obtained upon the homogenization of the viscera was submitted to a partial purification with ethanol. The protein content, proteolytic activity of the fractions thus obtained was assessed. The fraction presenting the highest proteolytic activity was further studied (30-70% of ethanol) resulting in 74.9% and 142.4% of protein recovery for tambaqui and carpa, respectively. This fraction was assayed at different temperatures and pH aiming to estimate the conditions for higher proteolysis. Temperarture and pH stability experiments were also carried out. Different commercially available detergents, surfactants and hydrogen peroxide were used to test the compatibility of these proteases with detergent formulations. The results reveal that these alkaline proteases extracted from *C. macropomum* show desirable characteristics for its use in laundry industry such as: retention of 73% and 63% of its initial activity in the presence of Surf® and 5% H_20_2, respectively, after 1 hour of incubation at 40ºC. In addition to that it was observed a slight increase of the proteolytic activity in the presence of non-ionic (Tween 20 and Tween 80) and ionic surfactants (Saponin and Sodium cholate). On the other hand, the proteases extracted from the intestine of *C. carpio* were compatible with the surfactants and oxidants tested but did not present the same property in the presence of commercially available detergents.

1 INTRODUÇÃO

Enzimas são catalisadores biológicos específicos, que aumentam extraordinariamente a velocidade das reações bioquímicas. Com exceção de uns poucos RNAs catalíticos, todas as enzimas conhecidas são proteínas (NELSON; COX, 2004). Como são essenciais, tanto para a manutenção, como para o crescimento e a diferenciação celular, essas moléculas são encontradas em todos os organismos vivos (GUPTA et al., 2002). Cada enzima é classificada de acordo com a reação específica que ela catalisa (NELSON; COX, 2004).

As proteases são enzimas que catalisam a hidrólise das ligações peptídicas entre as proteínas (BERG et al., 2004), podendo ser classificadas segundo o valor do pH no qual apresentam atividade máxima, diferenciando-se dessa forma em: proteases ácidas, neutras ou alcalinas (RAO et al., 1998).

Um aumento fenomenal no uso de proteases alcalinas como catalisadores industriais vem sendo registrado nos últimos anos, por oferecerem diversas vantagens em detrimento dos catalisadores químicos convencionais: possuem alta atividade catalítica, alto grau de especificidade pelo substrato, podem ser produzidas em larga escala, são economicamente viáveis e biodegradáveis (ANWAR; SALEEMUDDIN, 1998).

Tendo em vista as recentes tentativas de desenvolvimento de tecnologias ambientalmente corretas, as proteases vêm sendo amplamente utilizadas nas indústrias de detergentes, alimentos, farmacêutica, couro e em processos de bioremediação (RAO et al., 1998). Atualmente, as proteases são as principais enzimas industriais (JOHAVESLY; NAIK, 2001), sendo grande parte obtidas a partir de microrganismos (GODFREY; WEST, 1996).

Apesar de sua presença em diferentes indústrias, é na formulação de detergentes em pó que as proteases são mais utilizadas, ajudando na remoção de manchas de origem protéica (BANERJEE et al., 1999). Por serem, na sua maioria, extracelulares, proteases bacterianas têm sido as mais utilizadas para produção em larga escala nessa indústria. No entanto, há um

grande custo com filtração para obter-se uma preparação livre de microrganismos (PHADATARE et al., 1993).

Em teleósteos, as proteases são amplamente encontradas em suas vísceras, podendo ser tanto ácidas, no estômago, quanto alcalinas, nos cecos pilóricos e/ou no intestino (ZENDZIAN; BARNARD, 1967). Contribuindo, assim, para a degradação das proteínas da sua dieta, de forma que os aminoácidos e os peptídeos sejam melhor aproveitados (BEZERRA et al., 2001a).

As vísceras correspondem a aproximadamente 5% da massa total do peixe, tendo um grande potencial biotecnológico por serem fonte dessas enzimas digestivas (MARTINEZ; SERRA, 1989; GILDBERG, 1992; CANCRE et al., 1999). Segundo Asgeirsson et al. (1989) e Gildberg; Overbo (1990), o intestino de peixes contém cerca de 1 g de enzimas proteolíticas alcalinas (tripsina, quimiotripsina e elastase) por quilo.

As proteases digestivas de peixes são estudadas desde 1940 (NORRIS; ELAM, 1940). No entanto, existe uma defasagem de informações sobre proteases de peixes dulciaqüícolas de regiões tropicais, bem como de suas aplicações (BEZERRA et al., 2001b).

A atual piscicultura de água continental brasileira responde por aproximadamente 18% da produção total de pescados e está baseada, principalmente nos cultivos de tilápia, carpa e tambaqui (IBAMA, 2005).

A carpa (*Cyprinus carpio*), pertencente à família Cyprinidae, é um peixe de clima temperado tolerante a grandes variações de temperaturas (3º-35ºC), sendo bentopelágico, habitando tanto água doce quanto salobra, com pH variando de 7 a 7,5. Pode medir até 120 cm e pesar até 37,3 kg (IGFA, 2001). São onívoros, alimentando-se principalmente de insetos aquáticos, crustáceos, anelídeos, moluscos, macro e microalgas, mas principalmente no sedimento (SCOTT; CROSSMAN, 1973).

Cyprinus carpio é um peixe altamente comercial, tanto na pesca (em países europeus, asiáticos e indianos), como na aqüicultura e na piscicultura ornamental. É o segundo peixe exótico de mais importante para a aqüicultura brasileira e responsável por mais de 50 e 86% da produção aqüícola dos estado de Santa Catarina e Rio Grande do Sul, respectivamente (IBAMA,2005).

O tambaqui (*Colossoma macropomum*), pertencente à família Characidae e sub-família Serrasalminae, pode atingir até 108 cm e pesar até 30,0 kg. Assim como a carpa, o tambaqui também é bentopelágico, mas só habita águas doces, em uma maior variação de pH (5 a 7,8). Alimentam-se principalmente de zooplâncton, insetos, caramujos e folhas (LOVSHIN, 1995).

Colossoma macropomum é a espécie nativa mais cultivada no Brasil (IBAMA, 2005), sendo muito importante nessa atividade por viver em uma água pobre em minerais e ser muito resistente a doenças. Além disso, é uma das espécies mais consumidas na região Norte, apresentando ampla distribuição nos rios daquela região (VAL; ALMEIDA-VAL, 1995).

O aumento da poluição ambiental e reconhecimento de que o uso dos recursos biológicos possui limite têm enfatizado a necessidade de melhorar a utilização de subprodutos da indústria pesqueira (GILDBERG, 1992). A biotecnologia promove um meio de transformar esse material em valiosos produtos, como as enzimas, trazendo uma fonte alternativa para indústrias que utilizam catalisadores em seus processos (CASTILLO-YÁÑEZ et al., 2005).

Segundo Bezerra et al. (2001a), a grande quantidade de vísceras eliminada pelo setor pesqueiro torna as proteases de teleósteos viáveis para processamentos industriais específicos, principalmente na indústria de alimentos e detergentes. Dessa forma, otimiza o aproveitamento do pescado e, conseqüentemente, reduz o desperdício.

2 OBJETIVOS

2.1 Objetivo Geral

Aplicar as proteases alcalinas das vísceras de tambaqui e carpa na indústria de detergentes.

2.2 Objetivos Específicos

- Definir um protocolo de semi-purificação para proteases alcalinas de vísceras de peixes;
- Determinar o pH e a temperatura ótima de proteases alcalinas semi-purificadas de vísceras de tambaqui e carpa;
- Investigar a estabilidade dessas proteases em relação ao pH e a temperatura;
- Verificar a compatibilidade de tais proteases com detergentes comerciais, agentes surfactantes e oxidantes.

3 REVISÃO BIBLIOGRÁFICA

3.1 Piscicultura dulcícola nacional

O potencial do Brasil para o desenvolvimento da piscicultura de água doce é imenso, constituído por 5.500.000 hectares de reservatórios de águas doces, aproximadamente 12% da água doce disponível no planeta, clima extremamente favorável para o crescimento de organismos aqüícolas, terras disponíveis e ainda relativamente baratas na maior parte do país, mão-de-obra abundante e crescente demanda por pescado no mercado interno (IBGE, 2005).

Embora as pesquisas voltadas para o cultivo de organismos aquáticos tenham se iniciado na década de 30 do século passado, as mesmas só foram intensificadas a partir de 1970. A piscicultura comercial brasileira se firmou como uma atividade econômica no cenário nacional da produção de alimentos a partir de 1990, época em que nossa produção de pescado cultivado girava em torno de 25.000 toneladas/ano (FAO, 1997; VALENTI, 2000).

Em 2004, foram produzidas 180.730,5 toneladas de peixes, sendo a região Sul a maior produtora, responsável por 33% da produção nacional (Figuras 1 e 2). Os principais peixes na aqüicultura brasileira foram: a tilápia (69.078 t), a carpa (45.169,5 t) e o tambaqui (25.572 t), criados, principalmente, em sistema de cultivo intensivo (IBAMA, 2005).

O cultivo intensivo de peixes é realizado em viveiros projetados especialmente para este fim, possuindo sistemas de abastecimento e escoamento controlados e povoamento com peixes de valor comercial. As taxas de estocagem são programadas para uma criação comercial de alta produtividade e, com o intuito de aumentar o crescimento dos peixes usa-se, além da fertilização, a ração balanceada. Para a criação ser economicamente viável, a ração deve proporcionar elevada conversão alimentar capaz de promover um crescimento rápido, e o peixe, por sua vez, deve alcançar alto valor de mercado (VINATEA, 1997).

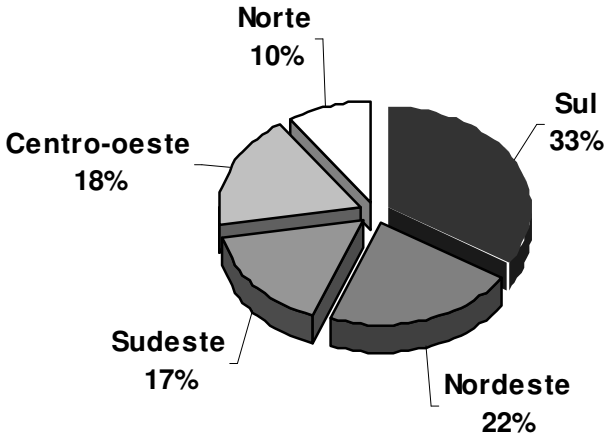

Figura 1: Porcentagem da produção brasileira de peixes dulcícolas por região em 2004. (Fonte: IBAMA, 2005).

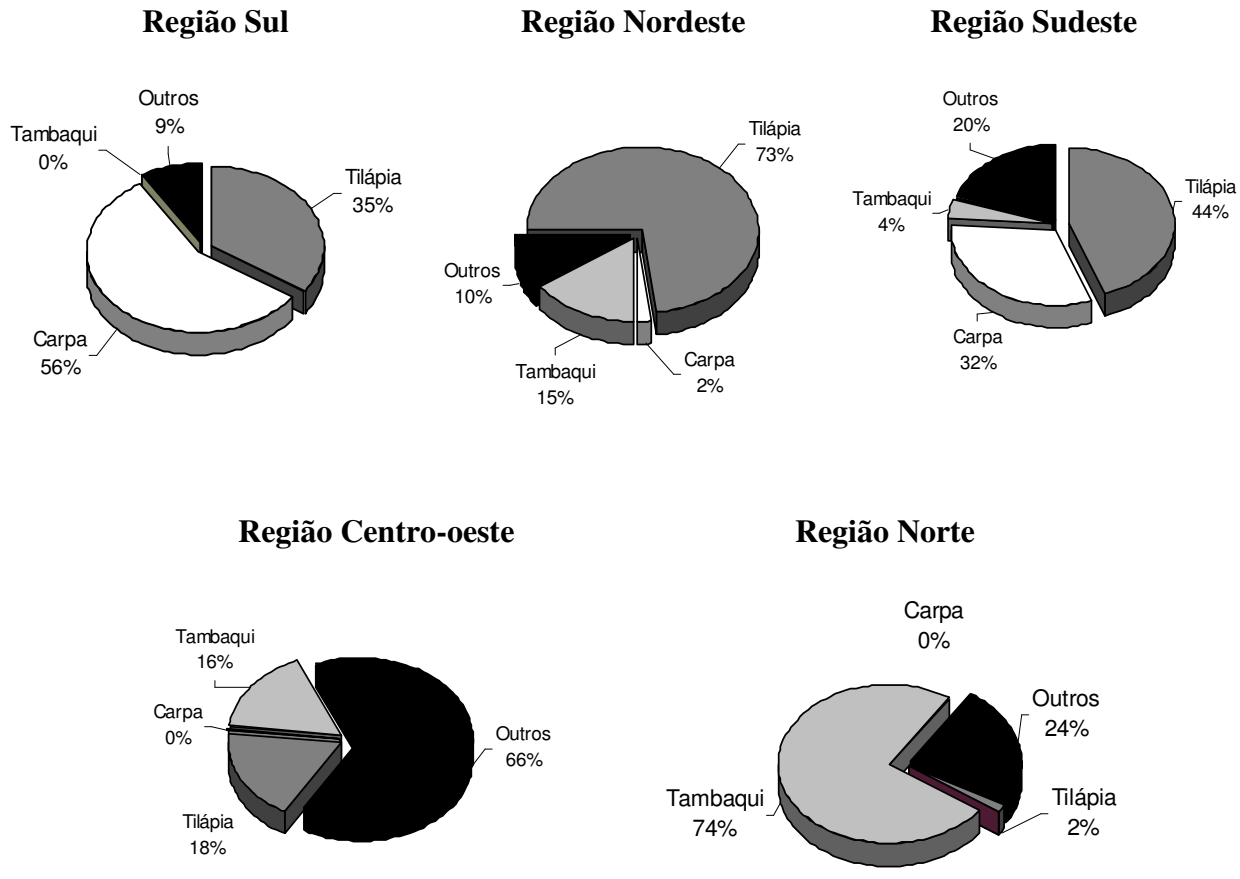

Figura 2: Produção de peixes de água doce em cada região do Brasil, destacando as três espécies mais cultivadas em 2004. (Fonte: IBAMA, 2005).

3.1.1 Tambaqui

O tambaqui, *Colossoma macropomum*, Cuvier, 1818, (Figura 3) pertence à família Characidae, sub-família Serrasalminae (NELSON, 1984). Esta espécie bentopelágica dulcícola nativa do Brasil está classificada taxonômicamente na seguinte forma, segundo Nelson (1984):

REINO Animmalia

SUB-FILO Vertebrata

SUPER-CLASSE Pisces ou Teleostomi

CLASSE Osteichthyes

SUB-CLASSE Actinopterygii

ORDEM Characiformes

FAMÍLIA Characidae

SUB-FAMÍLIA Serrasalminae

GÊNERO Colossoma

ESPÉCIE *Colossoma macropomum*, Cuvier, 1818

Figura 3: Tambaqui, *Colossoma macropomum*. (Foto: http://www.fishbase.org).

Os peixes pertencentes à sub-família Serrasalminae estão amplamente distribuídos pela América do Sul, sendo abundantes nas bacias dos grandes rios Amazonas, Paraná-Paraguai e Orinoco. Nestes rios, formam uma parte importante das capturas comerciais com fins de consumo humano e, em especial, as espécies dos gêneros *Colossoma* e *Piaractus* e a espécie *Mylossoma duriventris* (pacu) (FINK, 1978 apud MACHADO-ALLISON, 1982).

Outras espécies desse grupo de peixes, tais como as do gênero *Metynnis* são importantes na indústria de aquários. Finalmente, um grupo importante, tanto do ponto de visto biológico como econômico, são as piranhas. Seus hábitos vorazes e de formação de cardumes, transformam esses peixes em parte importante dos ecossistemas dulciaqüícolas sul americanos (FINK, 1978 apud MACHADO-ALLISON, 1982).

A característica mais relevante das espécies do gênero *Colossoma* é a presença de um grande número de cecos pilóricos, que variam de 30 a 40, mas podendo chegar até a 75 (HONDA, 1974 apud MACHADO-ALLISON, 1982) (Figura 4). Zedzian; Barnard (1967) sugerem que este órgão tem função similar ao pâncreas de outros vertebrados, responsáveis pela produção de proteases alcalinas. Em um estudo de caracterização de proteases extraídas dos cecos pilóricos do tambaqui (*C. macropomum*), Bezerra et al. (2000) encontraram proteases alcalinas termoestáveis, símilies à tripsina.

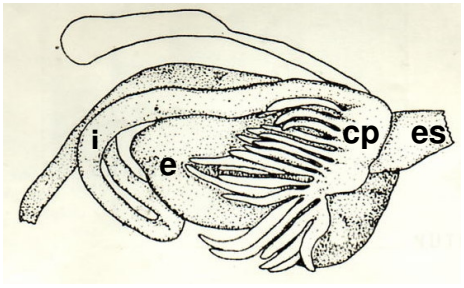

Figura 4: Vista lateral direita do sistema digestório do tambaqui. es - esôfago; e - estômago; i - intestino; cp - cecos pilóricos. (Fonte: MACHADO-ALLISON, 1983).

A alimentação principal do tambaqui é constituída por microcrustáceos planctônicos e frutas, ingerindo também algas filamentosas, plantas aquáticas frescas e em decomposição, insetos aquáticos e terrestres que caem na água, caracóis, caramujos, frutas secas e carnosas e sementes duras e moles (LOVSHIN, 1995).

Nos viveiros os tambaquis podem ser alimentados com frutas, tubérculos, sementes e rações peletizadas e extrusadas (VINATEA, 1997). O tambaqui alimenta-se rápido e agressivamente, não dando tempo para outros peixes comerem, no entanto, em sistema de policultivo pode ser cultivado junto com a curimatã, carpas e tilápia. Atinge peso médio de 1,5 kg em um ano de cultivo (HANCZ, 1993; TEICHERT-CODDINGTON, 1996).

3.1.2 Carpa

Cyprinus carpio, Linnaeus, 1758 (Figura 5) pertence à Classe Actinopterygii, Ordem Cypriniformes, família Cyprinidae, na qual são encontrados peixes de água doce ou salobra. A carpa está classificada taxonômicamente da seguinte forma, segundo Nelson (1984):

REINO Animmalia
SUB-FILO Vertebrata
SUPER-CLASSE Pisces ou Teleostomi
CLASSE Osteichthyes
SUB-CLASSE Actinopterygii
ORDEM Cypriniforme
FAMÍLIA Ciprinidae
GÊNERO Cyprinus
ESPÉCIE *Cyprinus carpio*, Linnaeus 1758

Figura 5: Carpa comum, *Cyprinus carpio*. (Foto: http://www.ceplac.gov.br).

Conhecido como carpa comum, *Cyprinus carpio* é a espécie de peixe doméstica mais importante do mundo e é cultivada há aproximadamente 4.000 anos (WOHLFARTH, 1993). É uma das quatro espécies sobre as quais existe um maior conhecimento científico e tecnológico de cultivo (CARVALHO et al., 2004; FRANCIS et al., 2002; HIDALGO et al., 1999; NANDEESHA et al., 2002; RITVO et al., 2004; RUANE et al., 2002; SALAM et al., 2005; WANG et al., 2005; YAMAMOTO et al., 2003).

No Brasil, é a espécie mais cultivada na principal região piscicultora, responsável por mais de 56% da produção do Sul (IBAMA, 2005). Suas características mais positivas são: a rusticidade, a capacidade de reprodução natural em cativeiro, o crescimento rápido, a aceitação de um amplo espectro de alimentos e o tamanho que atinge (PROENÇA; BITTENCOURT, 1994).

Embora considerada onívora, a carpa apresenta preferência por pequenos organismos animais. O primeiro alimento das larvas são rotíferos, seguidos de cladóceros. À medida que crescem, as carpas demonstram nítida preferência por organismos bentônicos, como larvas de quironomídeos, poliquetas e pequenos moluscos. Dependendo da disponibilidade destes organismos, a carpa pode ingerir detritos (nos quais bactérias e protozoários constituem-se nas principais fontes de nutrientes), sementes de plantas aquáticas e organismos zooplanctônicos (PROENÇA; BITTENCOURT, 1994).

Em um ano de cultivo atinge peso médio de 1,0kg (SCOTT; CROSSMAN, 1973). No sistema de policultivo, se adapta bem com o tambaqui, a carpa capim, a carpa prateada e a tilápia (SALAM et al., 2005).

3.2 Enzimas

As enzimas são as proteínas mais notáveis e especializadas, desempenhando o papel de catalisadores nas diversas reações bioquímicas. São fundamentais para a vida, pois sem a catálise, essas reações não ocorreriam em uma escala de tempo útil. Agindo em seqüências organizadas, elas catalisam as centenas de reações sucessivas pelas quais as moléculas nutrientes são degradadas, aumentando a velocidade das reações, sem afetar o seu equilíbrio (BERG et al., 2004; NELSON; COX, 2004).

As enzimas atuam de forma altamente específica com seus substratos. A reação enzimática ocorre no interior dos limites de uma cavidade na enzima chamada sítio ativo (Figura 6), onde a molécula que se liga a essa região é contornada com resíduos de aminoácidos cujos grupos substituintes se ligam ao substrato e catalisam a sua transformação (NELSON; COX, 2004).

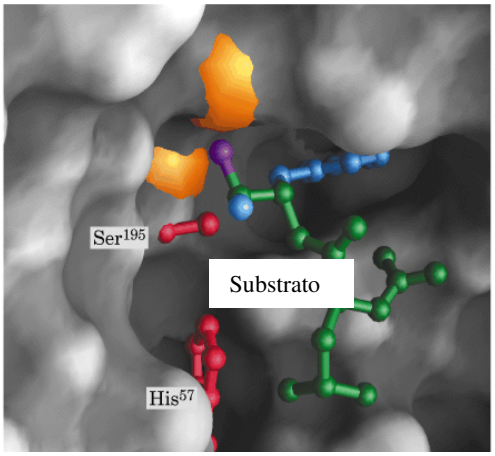

Figura 6: União de uma proteína ao sítio ativo de uma serinoprotease. (Fonte: NELSON;COX, 2004).

Uma vez que a reação química catalisada por uma enzima é a propriedade específica que distingue uma enzima de outra, a IUBMB dividiu as enzimas em seis grandes divisões (Tabela 1).

Tabela 1: Classificação das enzimas segundo a IUBMB.

CLASSE	REAÇÕES QUE CATALISAM
Oxidorredutases	Reações de oxidação-redução
Transferases	Reações de grupos contendo C, N ou P-
Hidrolases	Clivagem das reações adicionando água
Liases	Clivagem de C-C, C-S e certas ligações de C-N
Isomerases	Racemização de isômeros ópticos ou geométricos
Ligases	Formação de pontes entre C e O, S, N acoplados a hidrólise de fosfatos de alta energia.

C, carbono; N, nitrogênio; P^-, íon fosfato; S, enxofre; O, oxigênio.
(Fonte: NELSON; COX, 2004).

As enzimas são usadas há muitos anos como aditivos em diversos processos em diferentes atividades industriais. A enzima renina (ou quimosina), por exemplo, é usada na produção de queijo há aproximadamente 5000 anos a.C. (ROBINSON, 1987). Nativos da América Central usam há vários séculos folhas do mamão papaia (que contém papaína) para amaciar carnes (BERNHOLDT, 1975).

Atualmente, as enzimas vêm sendo largamente utilizadas nas indústrias de alimentos, têxtil, farmacêutica e de tratamento de resíduos, como uma ferramenta econômica e ambientalmente viável (ANWAR; SALEEMUDDIN, 1998; GUPTA et al., 2002; JOHNVESLY; NAIK, 2001).

3.2.1 Proteases

Por apresentarem diversas aplicações biotecnológicas, as proteases destacam-se entre as enzimas industriais. De acordo com a IUBMB as proteases estão inseridas no subgrupo 4 do grupo 3 (Hidrolases), pois por uma reação de hidrólise, elas clivam a proteína adicionando uma molécula de água à ligação peptídica (BERG et al., 2004) (Figura 7).

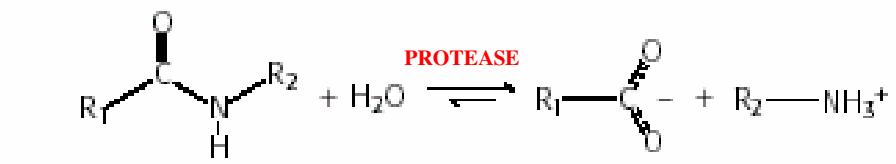

Figura 7: Hidrólise enzimática de uma proteína hipotética. (Fonte: BERG et al., 2004).

No entanto, as proteases não se adaptam tão bem a esse sistema geral de nomenclatura de enzima, pois apresentam uma grande variedade de estruturas e de ações. Comumente as proteases são classificadas com base em três principais critérios: (i) tipo de reação catalisada, (ii) natureza química do sitio catalítico e (iii) relação evolutiva com referencia a estrutura (BARETT, 1994).

A grosso modo, as proteases podem ser divididas em dois principais grupos: exopeptidases e as endopeptidases (Tabela 2). As do primeiro grupo atuam próximo das extremidades das cadeias e as endopeptidases atuam preferencialmente nas regiões internas das cadeias polipeptídicas (BARRETT, 1994; RAO et al., 1998). Neste segundo grupo encontram-se as principais proteases industriais (Tabela 3).

À parte de sua importância biológica, como ativação de zimogênios, transporte e composição do sangue, entre outras funções, as proteases são altamente relevantes no contexto biotecnológico (MAURER, 2004). A utilização de proteases na indústria é

responsável por aproximadamente 60% do mercado total de enzimas, entre estas, as alcalinas são as mais aplicadas (ANWAR; SALEEMUDDIN, 1998; GUPTA et al., 2002) (Figura 8).

Tabela 2: Classificação e divisão das proteases.

Local de clivagem no substrato	**Sítio ativo da enzima**	**Número de resíduos de aminoácidos removidos**
Exopeptidades	Aminopeptidases	Aminopeptidases Aminodipeptidases Aminotripeptidases
		Sítio ativo da carboxipeptidase
	Carboxipeptidases	Serinocarboxipeptidases Metalocarboxipeptidases Cisteínocarboxipeptidases
Endopeptidases	Serinoproteases Aspartatoproteases Cisteinoproteases Metaloproteases	

(Fonte: RAO et al., 1998)

Tabela 3: Classificação e uso de endopeptidases.

Grupo	**pH**	**Sítio Ativo**	**Exemplos de aplicações**	**Fontes**
Serinoproteases	Alcalino	Ser (His)	Hidrólise protéica	Tripsina (Pâncreas)
			Detergentes	Subtilisina (Bacillus)
Metaloproteases	Neutro	Zn^{2+}	Cerveja	*Bacillus*
		Ca^{2+}	Couro	*Aspergillus oryzae*
Carboxiproteases	Ácido	Asp	Queijo	Quimosina (bezerro)
Tiolproteases	Tiol	Cys	Amaciamento de carne	Papaína (Papaya)

(Fonte: RAO et al., 1998)

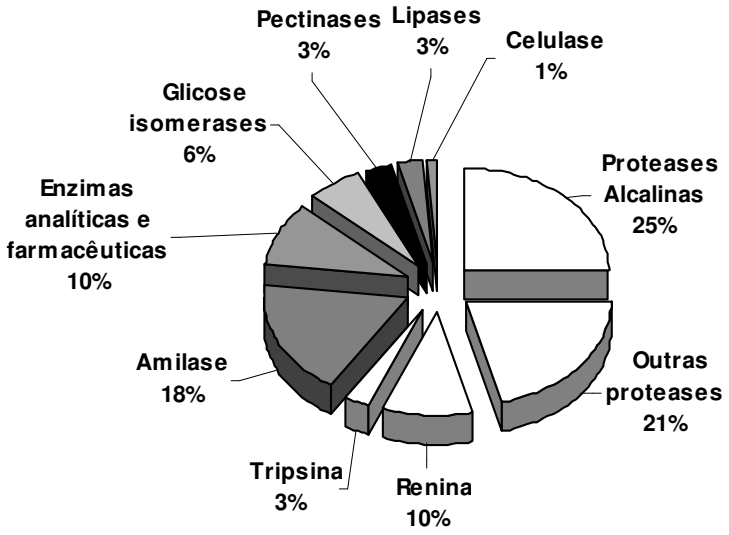

Figura 8: Enzimas no mercado mundial. (Fonte: RAO et al., 1998).

3.2.2 Proteases digestivas de peixes

Com o auxilio das proteases, as proteínas adquiridas na dieta são degradadas até que seus peptídeos e aminoácidos constituintes possam ser utilizados para a síntese de novas proteínas (BERG et al., 2004).

Em teleósteos, as proteases digestivas são amplamente encontradas nas suas vísceras, um dos principais resíduos deixados pela indústria pesqueira. A produção e excreção das proteases digestivas destes peixes ocorrem de forma muita parecida ao observado nos mamíferos (KOLODZIEJSKA; SIKORSKI, 1996).

O estômago secreta HCl e contém pepsina, uma protease que é produzida no epitélio sob a forma de pepsinogênio. Esse zimogênio é ativado por autocatálise, liberando cerca de 40 a 50 resíduos de aminoácidos da sua região N-terminal. As proteases digestivas do pâncreas são produzidas sob a forma de zimogênios, como o tripsinogênio, e são ativadas no lúmen do intestino pela ação da enteroquinase (enteropeptidase), uma protease do intestino

delgado, que hidrolisa uma ligação peptídica específica no tripsinogênio, transformando-o em tripsina ativa. A partir disto, as moléculas de enteroquinase juntamente com as de tripsina (recém ativadas) promovem um efeito cascata, responsável pela ativação de novos tripsinogênios e outros zimogênios como o quimiotripsinogênio, procarboxipeptidase, proelastase e profosforilase (BRODY, 1994). Segundo Glass et al. (1989), em algumas espécies, como entre os teleósteos, o pâncreas não é individualizado, encontrando-se difuso em outros órgãos, como nos cecos pilóricos.

O primeiro registro de estudo sobre proteases digestivas de peixes data da década de 40, quando uma pepsina de salmão foi cristalizada (NORRIS; ELAM, 1940). Desde então, proteases digestivas de peixes de águas temperadas vêm sendo comumente estudadas, incluindo não só as pepsinas, mas também as tripsinas, quimotripsinas, gastricsinas e elastases.

Pepsinas (E.C. 3.4.23.1) são proteases ácidas, encontradas no estômago dos animais. Estudos de isolamento e caracterização de pepsinas de peixes são comuns na literatura: *Katsuonus pelamis* (KUBOTA; ONHUMA, 1970), *Mustelus canis* (BAR-ELI; MERRETT, 1970), *Salvelinus fontinalis* (OWEN; WIGGS, 1971), *Sardinos melanostica* (NODA; MURAKAMI, 1981), *Merluccius gayi* (SANCHEZ-CHIANG; PONCE, 1982), *Mallotus villosus* (GILDBERG; RAA, 1983), *Salmo gairdneri* (TWINING et al., 1983), *Gadus mohua* (BREWER et al., 1984; GILDBERG et al., 1990, 1991; MARTINEZ, OLSEN, 1989), *Boreogadus saida* (ARUNCHALAM; HAARD, 1985), *Gadus ogac* (SQUIRES et al., 1986), *Oncorhynchus keta* (SANCHEZ-CHIANG et al., 1987), *Sebastes mentella*, *Sparus aurata, Scophthalmus maximus* (MUNILLA-MORÁN; SABORIDO-REY, 1996), Sparideos (DIAZ-LÓPES et al., 1998), *Takifugu rubripes* (KUROKAWA et al., 2005), reportando que as pepsinas são geralmente ativadas em valores de pH mais elevados e têm maior atividade específica que pepsinas de mamíferos.

Brewer et al. (1984) sugerem a utilização de pepsinas do bacalhau do Atlântico na fabricação de queijos do tipo Cheddar. Pepsinas desse peixe têm sido comercialmente produzidas para utilização como uma ferramenta em dissecações bioquímicas e para separação de tecidos biológicos, podendo ser também utilizadas na remoção da pele de peixes, como uma alternativa dos tratamentos mecânicos e químicos (DE VECCHI; COPPES, 1996).

As gastricsinas (EC 3.4.23.3), assim como as pepsinas, são aspatilproteases. Em estudo de purificação Sanchez-Chiang; Ponce (1981) purificaram dois zimogênios da gastricsina, encontrados na mucosa gástrica de *Merluccius gayi*. Essas proteases exibiram atividade máxima em pH 3,0, similar àquelas encontradas em humanos, porcos e sapos.

Em muitas espécies de peixes, tripsinogênios e zimogênios são secretados pelos cecos pilóricos. A atividade da tripsina (EC 3.4.21.4) em diferentes peixes de clima temperado vem sendo estudada: *Salmo gairdneri* (KITAMIKADO; TACHINO, 1960), *Gadus morhua* (ASGEIRSSON et al., 1989; BJARNASSON et al., 1993; OVERNELL, 1973), *Sardinos melanostia* (MURAKAMI; NODA, 1981), *Mallotus villosus* (HJELMELAND; RAA, 1982); *Protoptera aethiopicus* (DE HAEN et al., 1977), *Parasilurus asotus* (YOSHINAKA et al., 1984), *Gadus ogac* (SIMPSON; HAARD, 1984), *Tautogolabrus adsperus* (SIMPSON; HAARD, 1985), *Salmo solar* (STOCKNES; RUSTAD, 1995), *Oncorhynchus mykiss* (KRISTJANSSON, 1991), *Siganus canaliculatus* (SABAPATHY; TEO, 1995). O pH ótimo das tripsinas desses peixes é alcalino, similarmente ao encontrado em tripsinas de invertebrados e de outros vertebrados.

De acordo com Ritskes (1971) e Orejana; Liston (1981), a tripsina é um componente importante na preparação de arenques. Simpson; Haard (1987) comprovaram que quando adicionada no processo de aceleração da fermentação do arenque a tripsina do bacalhau do Atlântico provocou maior solubilização das proteínas quando comparado com a tripsina bovina. Outro uso de tripsina de bacalhau é na extração de carotenoproteínas de resíduos do

camarão, que também demonstrou ser mais eficiente que a tripsina bovina (CANO-LOPEZ et al., 1987).

Overnell (1973) foi o primeiro autor a relatar a atividade da quimotripsina (EC 3.4.21.4) em extratos brutos dos cecos pilóricos de *Gadus mohua*. Mas só em 1991 foram purificadas por Asgeirsson; Bjarnasson (1991). Kristjánsson; Nielsen (1992) isolaram quimotripsina dos cecos pilóricos de *Oncorhynchus mykiss*. As duas quimotripsinas isoladas tiveram pH ótimo de 7,8, similarmente ao encontrado em bovinos.

A elastase pancreática (EC 3.4.21.36) digere especificamente elastina em condições alcalinas. Essa enzima pertence à família das serinoproteases e vinha sendo estudada em poucas espécies de peixes: *Cyprinus carpio* (COHEN et al., 1981a,b), *Solea solea* (CLARK et al., 1985). Yoshinaka et al. (1985) caracterizaram elastase pancreática de *Thunnus thynnus*, *Seriola quinqueradiata*, *Anguila japonica* e *Lateolabrus*. Elastases de *Gadus mohua* foram as primeiras a serem purificadas e caracterizadas (RAA; WALTHER, 1989; GILDBERG; OVERBO, 1990). Anos depois, uma elastase intestinal de *Gadus mohua* foi purificada por Asgeirsson, Bjarnasson (1993) e Kristjánsson et al. (1995).

Recentemente, a caracterização e purificação de proteases alcalinas extraídas das vísceras de peixes tropicais vêm sendo realizadas. Os resultados destas pesquisas mostram proteases com características peculiares para aplicações biotecnológicas, principalmente na indústria de detergente, que requer proteases com pH ótimo elevado e termoestabilidade em temperaturas altas (Tabela 4).

Peixes de clima temperado, mas muito bem adaptados ao clima tropical do Brasil, como as carpas, também apresentaram características interessantes, como foi mostrado por Aranishi et al. (1998) quando da purificação de dipeptidades de *Cyprinus carpio*, onde estas tiveram maior atividade no pH 9,0 e a 60ºC. Apesar desse potencial já ter sido relatado desde a década de 80 por Cohen et al. (1981a,b) e Jónás et al. (1983).

A diversidade biológica dos peixes permite uma variedade de proteases com propriedades únicas (DE VECCHI; COPPES, 1996), fato que, aliado à grande quantidade de vísceras disponíveis no mercado, tornam as proteases desses teleósteos potencialmente viáveis para processos industriais específicos, principalmente nas indústrias de alimentos e detergentes (BEZERRA et al., 2001a).

Tabela 4: Estudos mais recentes de caracterização de proteases alcalinas extraídas das vísceras de peixes tropicais.

Ambiente	**Espécie**	**Propriedades**		**Autor/ano**
		pH ótimo	**Temperatura ótima (ºC)**	
Marinho	*Mugil cephalus*	8,0	50	Guizani et al.(1991)
	Siganus canaliculatus	8,0	55	Sabapathy; Teo (1995)
	Pseudupeneus maculatus *Caranx hippos* *Sparisoma* sp.	7,0-9,0	55	Alencar et al. (2003)
	Katsuwonus pelami *Thunnus albacores* *Thunnus tonggol*	9,0	55	Klomklao et al. (2004)
	Sardinops sagax caerulea	7,0-8,0	50	Castillo-Yánez et al. (2005)
	Pseudupeneus maculatus	9,0	55	Souza et al. (2006)
Dulcícola	*Oreochromis niloticus*	8,0	50	Bezerra et al. (2005)
	Tilapia nlotica/aurea	9,0	40	El-Shemy; Levin (1997)
	Colossoma macropomum	7,0-9,0	65	Bezerra et al. (2000)
		9,5	60	Bezerra et al. (2001b)
	Brycon orbignyanus	10	60	Garcia-Carreño et al. (2002)
	Hoplias malabaricus	7,0-9,0	55	Alencar et al. (2003)

3.2.3 Aplicação de proteases alcalinas na indústria de detergentes em pó

As proteases são um dos principais ingredientes de uma grande variedade de detergentes, desde aqueles usados para limpezas domésticas, àqueles usados para limpeza de lentes de contato ou dentaduras. Sendo que a maior parte dessas proteases é utilizada em detergentes em pó, respondendo por aproximadamente 25% do mercado total de enzimas (RAO et al., 1998).

Um pré-requisito para que enzimas proteolíticas possam ser usadas na formulação de detergentes é que elas sejam alcalinas e termostáveis, com um pH ótimo alto. Essas características são importantes devido ao pH do sabão em pó, que é geralmente entre 9-12 e a temperatura de lavagem que varia de 50 a 60ºC (TAKAMI et al., 1989; MANACHINI; FORTINA, 1998).

No entanto, existem outros fatores envolvidos na seleção de proteases para detergentes, como sua compatibilidade com o sabão em pó e os componentes presentes na sua fórmula, tais como agentes surfactantes, oxidantes, perfumes e alvejantes (KUMAR et al., 1998).

A preparação do primeiro detergente contendo enzimas data de antes de 1913, consistia de carbonato de sódio e um extrato pancreático bruto. O primeiro detergente contento enzimas bacterianas foi introduzido em 1956 (RAO et al., 1998). No entanto, a importância econômica das proteases alcalinas só surgiu quando proteases alcalinas de bactérias do gênero *Bacillus* foram introduzidas nos anos 60 para facilitar a liberação de material de origem protéica em manchas como aquelas de sangue, molhos, ovos e leite (KUMAR; TAKAGI, 1999; GUPTA et al., 2002).

A adição de enzimas proteolíticas alcalinas na formulação de detergentes aumenta consideravelmente o potencial de limpeza entre 30 e 40% (MOREIRA et al., 2002). O

benefício do uso destas enzimas envolve também a conservação das fibras dos tecidos e seu caráter biodegradável.

Atualmente, a enzima utilizada em todos os detergentes em pó é a subtilisina, uma serinoprotease (US patente nº 1240058, 374971, 370482, e 4266031, e UK patente nº 13155937). Em 2002, a União Européia produziu e usou cerca de 900 toneladas dessas proteases (MAURER, 2004). Apesar de ser adicionada até os dias atuais, ela não é uma protease ideal para este fim, devido a sua baixa estabilidade térmica na presença de detergente e curta vida de prateleira (SAMAL et al., 1990), além de requerer metodologias de filtração de custo intensivo para obter preparação de enzimas livre de microrganismos.

A utilização de proteases de fungos tem sido proposta por alguns autores (SAMAL et al., 1990; PHADATARE et al., 1993; MOREIRA et al., 2002). Apesar de oferecerem a vantagem de seu micélio ser facilmente removido por filtração, para que haja uma produção economicamente viável é necessário otimizar o meio de fermentação (PHADATARE et al., 1993). Por esta razão, é essencial buscar novas fontes de protease (BENERJEE et al., 1999).

Existem duas formas de se conseguir enzimas estáveis para o trabalho industrial: através da engenharia genética, submetendo colônias recombinantes a mutações (WELLS; POWERS, 1986; PANTOLIANO et al., 1987; VON DER OSTEN et al., 1993) ou simplesmente buscando novas fontes de enzimas que sejam estáveis em extremos de pH, temperatura e na presença de desnaturantes como uréia e detergentes (COWAN; DANIEL, 1982; WOJTCZAK, 1987; TAKII et al., 1987).

3.3 Purificação de proteínas

O processo de purificação de uma proteína requer primeiramente a separação desta dos componentes celulares. Os tecidos e células são rompidos em solução tampão, obedecendo a

certos critérios que evitam a desnaturação da proteína de escolha, de modo que se forma uma mistura denominada extrato bruto (BRACHT; ISHII-IWAMOTO, 2002). Estas biomoléculas podem ser purificadas de acordo com diferentes métodos que se baseiam em diferenças físicas como, tamanho da molécula protéica, carga elétrica e afinidade com outras moléculas (NELSON; COX, 2004).

3.3.1 Purificações baseadas no tamanho da molécula

A primeira etapa em um típico protocolo de purificação de proteína é a centrifugação. O princípio deste método é que diferentes partículas em suspensão (células, organelas ou moléculas), tendo diferentes massas ou densidades, estabelecer-se-ão no fundo de diferentes índices (DEVLIN, 1998). A centrifugação diferencial separa proteínas solúveis de materiais insolúveis; a força centrífuga e a duração da centrifugação são ajustadas para assegurar que os materiais insolúveis sedimentem, formando precipitados, de forma que as proteínas solúveis permaneçam no líquido sobrenadante. As proteínas aí contidas podem ser então separadas por outros métodos de purificação (NELSON; COX, 2004).

As proteínas globulares em solução podem ser separadas facilmente de solutos de peso molecular baixo, por diálise, a qual utiliza uma membrana semipermeável para reter as moléculas protéicas permitindo a passagem das moléculas menores de soluto e água. A ultrafiltração é um método que usa a força centrífuga ou a pressão para filtrar o meio aquoso e as moléculas pequenas de soluto através de uma membrana semipermeável que retém moléculas protéicas (NELSON; COX, 2004).

3.3.2 Purificações fundamentadas nas diferenças de solubilidade

As proteínas em solução apresentam alterações profundas na solubilidade em função do pH, força iônica, propriedades dielétricas do solvente e temperatura (CHAMPE; HARVEY, 1996).

Proteínas podem ser separadas em função de seu ponto isoelétrico ou ponto isolítico, definido como o pH em que a molécula não apresenta carga elétrica efetiva e é incapaz de migrar ou deslocar-se num campo elétrico. Misturas de proteínas em pH ajustado para o seu pH isoelétrico precipitam e não perdem a conformação nativa, podendo ser ressuspensas em um meio que apresente pH adequado e concentração de sal conveniente (VOET; VOET, 2005; NELSON; COX, 2004).

Uma forma de reduzir a solubilidade de proteínas se faz pelo uso de solventes orgânicos, como o etanol e a acetona. O uso desses compostos pode desnaturar proteínas se usado a elevadas temperaturas e, por isso, o controle da temperatura quando se usa essa técnica deve ser considerado. A maioria das proteínas expostas a temperaturas na faixa de 40 a 50°C começam a desnaturar e perdem solubilidade, constituindo outro parâmetro de isolamento dessas biomoléculas (NELSON; COX, 2004; CAMPBELL, 2001).

Muitos solventes orgânicos miscíveis em água são capazes de precipitar enzimas. Devido a sua baixa constante dielétrica (quando comparado com a água), solventes orgânicos aumentam a atração entre as moléculas de proteínas, formando agregados, até que as partículas assumam proporções macroscópicas e precipitem. Este fenômeno consiste na remoção da água de solvatação da proteína, permitindo que forças eletrostáticas induzam regiões de cargas opostas da proteína a se atraírem. Neste caso, a água é removida tanto pelo solvente orgânico, como pela estruturação ao redor da molécula orgânica. Como

conseqüência, a constante dielétrica é diminuída (SCOPES, 1988; WANG et al., 1979; HARRISON, 1993).

Os álcoois - metanol, etanol e isopropanol - são os mais importantes precipitantes industriais. O etanol, no entanto, apresenta o balanço ideal entre o efeito na solubilidade e características hidrofóbicas adequadas para reduzir a desnaturação. A precipitação com etanol é uma técnica promissora que pode ser aplicada para muitos tipos de proteínas em escala industrial. O etanol é, depois da água, o mais importante dos solventes, por possuir boas características físico-químicas, como uma completa miscibilidade com a água, baixo ponto de fusão, ausência de risco de misturas explosivas, alta volatilidade, inércia química, baixa toxicidade e baixo custo, especialmente no Brasil (CORTEZ; PESSOA Jr., 1999).

Os sais neutros têm efeito pronunciado sobre a solubilidade de proteínas. Para Nelson; Cox (2004) os sais de íons divalentes, tais como $MgCl_2$ e $(NH_4)_2SO_4$, são muito mais eficientes na solubilização do que os sais de íons monovalentes como o NaCl, NH_4Cl e KCl. Com o uso dos sais ocorre o aumento de solubilidade (*salting in*) ou perda de solubilidade (*salting out*) das proteínas. O sulfato de amônio é o sal mais usado para a precipitação, pois tem solubilidade acentuada e produz força iônica elevada (BRACHT; ISHII-IWAMOTO, 2002).

Bezerra et al. (2001b) apresentaram um método para purificação de enzimas do tipo tripsina de tambaqui (*Colossoma macropomum*), baseado na termoestabilidade dessa enzima e composto de três etapas: tratamento térmico, fracionação com sulfato de amônio e filtração em gel Sephadex. O tratamento térmico provou ser uma estratégia eficiente na purificação de proteases de peixes, por desnaturar e remover proteínas não resistentes ao calor ainda no extrato bruto, no entanto, a indústria tem restrições em relação ao sulfato de amônio, que é corrosivo e desgasta mais rapidamente os materiais metálicos utilizados nos processos industriais (MARCO, 2005).

3.3.3 Purificações baseadas na carga elétrica

Os processos desse tipo de separação dependem, em última análise, das propriedades ácido-básicas das proteínas, que são grandemente determinadas pelo número de tipos de grupamentos "R" ionizáveis de suas cadeias laterais (NELSON; COX, 2004).

A eletroforese é uma técnica de separação de moléculas em uma mistura sob influência de um campo elétrico aplicado. As moléculas dissolvidas no campo elétrico se movem ou migram em uma velocidade determinada por sua carga: razão de massa. As determinações de pureza e do peso molecular da biomolécula purificada são feitas mais comumente por eletroforese em gel de poliacrilamida em condições desnaturantes (BRACHT; ISHII-IWAMOTO, 2002).

Em uma das formas de eletroforese em gel de poliacrilamida (PAGE), o detergente dodecilsulfato de sódio (SDS) é usado para desnaturar as proteínas. Moléculas anfifílicas, como o SDS, interferem nas interações hidrofóbicas que normalmente estabilizam as proteínas. As proteínas em geral assumem uma forma cilíndrica na presença de SDS. Além disso, a maioria das proteínas liga-se ao SDS na proporção de 1,4g de SDS por grama de proteína (cerca de uma molécula de SDS para cada dois resíduos de aminoácido). A carga negativa que o SDS transfere mascara a carga intrínseca da proteína. O resultado líquido é que as proteínas tratadas com SDS possuem formas similares e razões carga/massa parecidas. Em conseqüência disso, a SDS-PAGE separa as proteínas por efeito de filtração em gel, isto é, de acordo com a massa molecular (VOET; VOET, 2005).

As massas moleculares das proteínas são rotineiramente determinadas pela SDS-PAGE com exatidão de 5 a 10%. A mobilidade relativa das proteínas em tais géis varia linearmente com o logaritmo das suas massas moleculares. Na prática, a massa molecular de uma proteína fazendo-se sua eletroforese em presença de proteínas marcadoras, de massa

molecular conhecida, e cobrindo uma faixa onde amassa molecular da proteína de interesse esteja situada. Pelo fato de o SDS romper as interações não-covalentes entre os polipeptídios, a SDS-PAGE fornece a massa molecular das subunidades das proteínas feitas de múltiplas subunidades. A possibilidade de as subunidades serem ligadas por pontes dissulfeto pode ser testada por meio da realização da SDS-PAGE na presença e na ausência de agentes redutores, tais como o 2-mercaptoetanol ($HSCH_2CH_2OH$), que quebra essas ligações (VOET; VOET, 2005).

3.3.4 Purificações fundamentadas na separação por adsorção seletiva

A cromatografia de afinidade baseia-se no principio de que as proteínas podem ser separadas de acordo com a sua capacidade de se ligar de forma não-covalente a outra molécula. Esse tipo de cromatografia apresenta ligantes que podem ser substratos enzimáticos ou outras moléculas, como anticorpos. A coluna de afinidade reage quando a proteína de interesse se liga ao ligante, sendo que as outras proteínas passam livremente pela coluna. As proteínas ligadas à coluna são, então, decantadas pela adição de excesso do ligante ou pela mudança na concentração de sal ou pH (BRACHT; ISHII-IWAMOTO, 2002; DEVLIN, 1998).

Os parâmetros de solubilidade para separar proteínas são usados, sobretudo nas fases iniciais de purificação protéica, mas eles não fornecem a resolução elevada dos métodos cromatográficos e eletroforéticos, bem mais precisos em relação às impurezas remanescentes (DEVLIN, 1998).

A cristalização das proteínas pode ser uma etapa seguinte de purificação. Este processo não é necessariamente um sinal de pureza completa, uma vez que os cristais protéicos podem conter contaminantes (NELSON; COX, 2004).

Não existe uma seqüência exata dos métodos de purificação a serem usados em todas as proteínas. Devlin (1998) e Voet; Voet (2005) relatam que deve-se escolher uma seqüência de técnicas de purificação que resulte em um elevado grau de purificação e alto rendimento. A obtenção de métodos sensíveis e específicos para distinguir e medir quantitativamente a proteína que se pretende isolar é também indispensável.

Vários testes são usados para detectar, identificar e quantificar proteínas. Os testes mais sensíveis usam uma reação que produz luz ou radioatividade para gerar um sinal. Os anticorpos são poderosos reagentes usados para essa finalidade. Eles são usados na cromatografia de afinidade e combinados com a eletroforese em gel na técnica de *Western blotting*, um eficaz método para a separação e detecção de uma proteína numa mistura (DEVLIN, 1998).

A autoradiografia é uma técnica semi-quantitativa para detecção de moléculas marcadas radioativamente nas células, tecidos ou géis de eletroforese (BRACHT; ISHII-IWAMOTO, 2002). As estruturas tridimensionais são obtidas por cristalografia pelos raios X, espectroscopia por ressonância magnética nuclear (RMN) e microscopia crioeletrônica. A cristalografia em raios X fornece estruturas mais detalhadas, mas requer a cristalização da proteína. A microscopia crioeletrônica é particularmente útil para grandes complexos protéicos, os quais são difíceis de cristalizar. Apenas proteínas relativamente pequenas são receptíveis à análise por RMN (DEVLIN, 1998; VOET; VOET, 2005).

Tropical fish alkaline proteases as a laundry detergent additive

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Process Biochemistry

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Running title: Fish proteases as a detergent additive

Abstract

Proteases were extracted from tambaqui (*Colossoma macropomum)* intestines, an Amazonian fish that is the most important native species to Brazilian aquaculture, followed by precipitation with ethanol inside the saturation of 30-70% (v/v). The clear preparation, without the typical fish smell, contained about 75% of the initial enzymes and presented at least five proteases according to the zymogram. The optimum pH was in the alkaline range of 10-12 inside that was stable for 30 min. The optimum temperature of activity was at 60^{o}C and only about 15% of the initial activity was lost after incubated for 30 min at this temperature. Trypsin and chymotrypsin-like enzymes, both serine proteases, were recognized among the proteases with higher prevalence of the first one. Tambaqui intestines proteases precipitated by ethanol were also stable in the presence of non-ionic (Tween 20 and Tween 80) and ionic surfactants (Saponin and Sodium choleate), except SDS that inactivated about 85-90% of their activities. Furthermore, they were incubated with high concentrations of H_2O_2 (10% v/v) and after 75 min they still retained 53.33% of the initial activity. They retained more than half of their activities in the presence for 1 h at 40^{o}C of several commercial detergents (Ala®, Bem-te-vi® and Omo®) and about three quarters in the presence of Surf®.

Keywords: Alkaline proteases, Tropical fish, Tambaqui (*Colossoma macropomum*), Laundry detergent, Surfactants, Oxidants agents.

1. Introduction

Proteases constitute one of the most import groups of industrial enzymes, accounting for at least 60% of the total global enzyme production sales [1-3]. The detergent industry has now emerged as the major consumer of several hydrolytic enzymes acting at highly alkaline pH. The major industrial use of detergent-compatible proteases is in laundry detergent formulations, these enzymes account for at least a quarter of the total protease sales in the world [3-6]. These applications have grown substantially. The increased usage of these enzymes as detergent additives is mainly because (a) they are biodegradable and (b) an increased performance/cost ratio was observed when these molecules where used in detergent formulations [3,6].

Alkaline proteases from bacteria, fungi or insect origin can be exploited commercially [5]. Although bacterial proteases have long been used in detergents, the main drawback in their use is that they require cost-intensive filtration methodologies to obtain a microrganism-free enzyme preparation. On the other hand, the proteases of fungal origin offer an advantage in that the mycelium can be easily removed by filtration, but to obtain high and commercially viable yields of proteases, it was essential to optimize fermentation medium for growth and production of proteases [7,8].

Moreover although Subtilisin have been the enzymes of choice for detergent formulations (US patent nos. 1240058, 374971, 370482 and 4266031 and UK patent nos. 13155937) they are not ideal detergent enzymes due to low thermal stability in presence of detergents and short half-life on the shelf [7]. Thus is desirable to search for new proteases with novel properties from many different sources as possible [9]. These properties have already been found in trypsin-like enzymes from tropical fishes [10-14].

Acid from stomach and alkaline from intestine are the most important digestive proteases of fish viscera, accounting for about 5% of total mass, which have wide

biotechnological potential as a source of digestive enzymes, especially proteases [15-19]. In general, tropical fish proteases have thermal stability, long shelf life and high activity over a wide pH range [10-14].

The contamination on account of wastes generates of the fish's processing is a challenge to be satisfactory solved. For instance, the Monterey sardine in Mexico involves tons of fish processing annually and generates wastes, mainly stick-water, viscera, heads and processing effluents, which are not treated and are discarded directly to the sea, causing pollution [20]. Therefore, alternatives, including commercial uses for the by-products and wastes, are urgently needed.

Tambaqui (*Colossoma macropomum*) is the most important native fish species of the Brazilian aquaculture, with an estimated production of about 25,000 tons in 2004 [21]. The proteases from this tropical fish have already been studied and those from stomach and pyloric caeca crude extracts were characterized [10] and a trypsin-like enzyme was identified [11]. Large amounts of this trypsin-like enzyme could be recovered from the tambaqui wastes based on the information that 2.7g was found per kg of pyloric caeca [11]. Furthermore, previous reports showed that the one kg of cod intestines produces one gram of trypsin-like enzyme [17,22]. Estimating a digestive-tract fraction as 5% of the total weight high quantities of digestive enzymes could be produced by appropriated processing use of the fish waste.

The present paper describes the extraction, partial purification and characterization of alkaline proteases from *C. macropomum,* as well as, its compatibility with commercial laundry detergents, oxidants and surfactants agents.

2. Materials and methods

2.1. Enzyme extraction

According to Bezerra et al. [13] intestines of *Colossoma macropomum* were collected and homogenized (20 mg per mL of tissue/ml in 0.9%, w/v, NaCl) by using a tissue homogenizer. The resulting preparation was centrifuged at 10,000 x*g* for 10 min at 10ºC to remove cell debris and nuclei. The supernatant (crude extract) was frozen at – 20ºC and used for purification steps.

2.2. Enzyme partial purification

The crude extract was incubated at 45ºC for 30 min and centrifuged at 10,000 x*g* for 10 min at 4ºC. Then the supernatant (300 mL) was fractionated with iced ethanol [23] to attain saturations from 0 to 30% (Fraction F_1) and from 30 to 70% (Fraction F_2). The ethanol (4^oC) was slowly added to the medium under mild stirring at 4ºC, the mixture was left to rest for 2 h and centrifuged at 10,000 x*g* 4ºC for 15 min. The pellets (Fraction F1 and F2) were dissolved in 75 ml 0.1 M Tris-HCl buffer (pH 8.0) at 25ºC and dialyzed against 4 liters of 0.05 M Tris-HCl buffer (pH 8.0) for 2 h. After which the buffer was renewed and additional dialysis occurred for 2 h and, finally, after a third buffer change dialysis was allowed to proceed overnight.

2.3. Enzymatic assay

Proteolytic activities were determined in the crude extract and in the fractions using 1% azocasein as substrate according to Alencar et al. [12]. A unit (U) of enzymatic activity was defined as the amount of enzyme capable of hydrolyzing azocasein to produce 0.001 change in absorbance per min. The protein content was estimated by measuring sample

absorbance at 280 and 260 nm by using the following equation: [protein] mg/mL = A280 nm X 1.5 - A260 nm X 0.75 [24]. The fraction presenting highest specific activity was chosen for compatibility and characterization studies.

2.4. Electrophoresis

The dialyzed enzyme (100µg of protein) was concentrated by liophilization and used for electrophoresis according to Laemmli [25], using 4% (w/v) stacking gel and 12.5% (w/v) separating gel. The gel was stained with 0.1% (w/v) Coomasie Blue for 2 h and destained in 10% (v/v) acetic acid and 25% (v/v) methanol. Bovine serum albumin (66 KDa), ovoalbumin (45 KDa), glyceraldehydes 3-phosphate dehydrogenase (36 KDa), carbonic anhydrase (29 KDa), trypsinogen (24 KDa) and α – lactoalbumin (14.2 KDa) were used as molecular weight standard. The zymogram was adapted from Garcia-Carreño et al. [26] and carried out as follows: after electrophoresis, the gel was immersed in 50 mL of 3% casein in 0.1 M Tris-HCl buffer, pH 8.0, for 30 min at 5ºC. Then the temperature was raised to 25ºC and further incubation for 90 min was allowed for the digestion of the protein substrate (casein) by the active fractions. The gels were then stained with 0.1% (w/v) Coomasie Blue for 2 h and destained in 10% (v/v) acetic acid and 25% (v/v) methanol.

2.5. Effect of inhibitors

Inhibition was measured according to Alencar et al. [12] and Bezerra et al. [13]. Enzyme extracts were incubated during 30 min with different specific protease inhibitors (8mM), such as the serine-protease inhibitor PMSF (phenyl-methyl-sulphonyl-fluoride), chymotrypsin-specific inhibitor TPCK (tosylamido-2-phenylethyl chloromethyl ketone), trypsin-specifics TLCK (tosyl-lysine chloromethyl ketone) and Benzamidine. After incubation, 8 mM trypsin and chymotrypsin specific substrates, BApNA (benzoil arginine p-

nitroanilyde) and Suc-Phe-p-Nan (succinyl phenylalanine p-nitroanilyde), were added. The release of *p*-nitroanilide was followed by increase in absorbance at 405 nm. The enzyme and substrate blank were similarly assayed without enzyme and substrate solution, respectively. The 100% values were established without any of the inhibitors.

2.6. Effect of pH

Protease activity was measured at different pH values under standard assay conditions with azocasein as a substrate. The enzymatic activity was assayed at pH 6.5-12.5 with 0.1 M phosphate buffer (pH 6.5-7.5), 0.1 M Tris-HCl buffer (pH 7.2-9.0) and 0.1 M NaOH/glycine buffer (pH 8.6-12.5). The effect of pH on the stability of the enzyme preparation was studied by incubating the enzyme at 25ºC for 30 min with the above buffers and then the enzymatic activities measured as described above at 25ºC, using 0.1 M NaOH/glycine buffer (pH 11.5).

2.7. Effect of temperature

The protease activity was assayed at various temperatures (25-80ºC) to determine the optimum temperature, at pH 8.0. For the thermal stability determination, previously to enzymatic activity, the enzyme preparation was incubated for 30 min at the temperature ranging from 25^{o}C to 80^{o}C. After the enzyme preparation temperature reached 25ºC their proteolytic activities were assayed.

2.8. Effect of oxidizing agent and surfactants

The hydrogen peroxide stability of the proteases from tambaqui precipitated by ethanol was investigated by incubating samples (600µL) with H_2O_2 (600 µL) at concentrations of 5%, 10% and 15% at 40º C. Samples (150 µL) were withdrawn at 15, 30 and 75 min to have their activities (duplicates) on azocasein established and compared to the

non-treated sample. Stability towards ionic (Saponin and Sodium choleate) and non-ionic surfactants (SDS, Tween 20 and Tween 80) was investigated by incubation at solution concentrations of 1% (w/v) for 30 and 60 min at 40ºC after which the enzyme activity was assayed [27].

2.9. Compatibility with commercial detergents

The protease at a concentration of 0.20 $mg.mL^{-1}$ was incubated at 40ºC with commercially available detergents: Ala® (Protec & Gamble); Bem-te-vi® (Alimonda); Omo Multi Ação® (UniLever) and Surf® (UniLever) to a final concentration of 7 $mg.mL^{-1}$. Samples (150 µL) were removed after a time interval of 10 min (total period of 60 min). The residual proteolytic activity in each sample was determined at 25ºC, assayed and compared with the control sample incubated at 40ºC without detergent pH 11.5 [27].

3. Results and discussion

The highest enzymatic recovery was observed in the Fraction F2 (30-70% of ethanol saturation), presenting purification and yield of 1.3 and 74.9%, respectively (Table 1). Although the low purification results provided by the ethanol precipitation this protocol allowed concentrating the enzyme as well as removed the typical fish smell from the preparation. The electrophoresis and zymogram of the crude extract and the Fraction F2 are presented in Figure 1. Standard protein markers of different molecular weights and the proteins of the crude extract and Fraction F2 are displayed in the lane 1, lane 2 and lane 3, respectively. Their proteolytic activities on casein are also shown (zymogram) in the lane 4 (crude extract) and lane 5 (Fraction 2). As can be observed in this figure the ethanol precipitation protocol was able to purify partially and concentrate three major proteins with

proteolytic activity from the crude extract. They showed molecular weights of 40.8, 35.5 and 22.7 kDa and two other small proteases presenting molecular weights between 14 kDa and 22.7 kDa. Mixture of proteases rather than purified ones are usually used in the food and detergent industries [1].

The effect of pH on the proteolytic enzyme is shown in Figure 2. The proteases of the ethanol Fraction F2 obtained from the tambaqui intestine displayed their maximum activities at the pH range of 10-12. The Tambaqui proteases were also stable over the pH range 10-12.5 (Figure 3) that coincides with found for the optimum pH range (10-12). One of the most important parameters for selection of proteases for detergents is the pH value. It is desirable that the pH of detergent solution in which proteases work should be approximately the same as the pH optimum for the enzyme [3]. Protease and other enzymes currently used in detergent formulations should be alkaline in nature with a high pH optimum because the pH of laundry detergents is commonly in the range of 9.0–11.0 [9]. These properties were achieved for the tambaqui proteases purified by ethanol precipitation.

Figure 4a shows the temperature effect on the tambaqui proteases activities and an optimum activity was found at 60º C. Also, they showed to be thermal stable because they retained about 91% and 86% of the activity after being incubated at 50ºC and 60ºC, respectively, for 30 min (Figure 4b). This parameter is similar to that described for proteases from *Bacillus brevis* [9].

All detergent-compatible enzymes are alkaline with a high optimum pH and thermo stable. These characteristics are important because the pH of laundry detergents is generally in the range of 9.0-12.0 and the thermal stability of these alkaline enzymes generally varies from 50 to 60ºC [27-29].

The use of specific substrate and protease inhibitors were used to identify the presence of trypsin-like and chymotrypsin-like enzymes in the ethanol-precipitated proteases from the

tambaqui intestines (Table 2). Proteases acting on BApNA were strongly inhibited Benzamidine and TLCK, classical trypsin-like inhibitors, whereas those hydrolyzing Suc-Phe-p-Nan were strongly inhibited by TPCK, typical chymotrypsin inhibitor. However, The results suggest that trypsin-like enzyme is the major protease in the preparation (about 40 times higher). The BApNA and Suc-Phe-p-Nan proteolysis were, respectively, inhibited 54% and 96% by PMSF, serine protease inhibitor. Similar results are frequently observed to other tropical fish proteases [12,13].

There are many parameters involved in the selection of a protease for detergents, such as compatibility with detergents components, e.g., surfactants, perfumes and bleaches [30-32]. In this study the proteases of tambaqui were assayed in the presence of all non-ionic (Tween 20 and Tween 80) and ionic surfactants (Saponin and Sodium choleate) using azocasein as substrate. The results are displayed in Table 3 and they did not loose activities after being incubated for 60 min with these reagents surfactants. Saponins are glycosides of steroids, steroid alkaloids (steroids with a nitrogen function) or triterpenes found in plants whereas sodium choleate is natural anionic surfactant bile salts derivative from cholesterol. SDS (Sodium dodecyl sulfate) was the unique detergent capable to inactivate them. It is an ionic surfactant that is widely used in many applications that has a tail of 12 carbon atoms, attached to a sulfate group, giving the molecule the amphiphilic properties required for a detergent.

Important commercial detergent proteases available, such as Subtilisin Carlsberg, Subtilisin BPN, Alcalase, Esparase and Savirase are stable in the presence of various components of detergents; however, the majority of them are unstable in the presence of peroxide agents [32]. Figure 5 presents the oxidant stability of the tambaqui proteases under the presence of hydrogen peroxide. The peroxide inactivation curve shows that they are stable even at high concentrations of H_2O_2 (10% v/v). After incubation of 75 min they retained 53.33% of the residual activity (initial activity without hydrogen peroxide). This is an

important property because the bleach stability has been attained only by site directed mutagenesis [33-35] or protein engineering [36, 37] on bacterial enzymes.

An ideal detergent enzyme should be stable and active in the detergent solution for a long period of time and should have adequate temperature stability to be effective in a wide range of washing temperatures [27, 38]. As seen Figure 6, the tambaqui proteases retained more than half of their activities in the presence of the detergents Ala®, Bem-te-vi® and Omo® for 1 h at 40^{o}C. The maximum stability was observed for the Surf®, as the enzyme retained 73.70% of its activity. Some studies regarding compatibility of proteases in the presence of detergents, showed activity retention of 64% and 90% to the fungi *Conidiobolus coronatus* and *Nocardiopsis* sp, respectively [8, 27], more than 70% on alkaline protease from *Bacillus cereus* [38] for 1 h at 40ºC and more than 60% at 60ºC from *Bacillus brevis* [9] for 1 hour.

Proteases should be effective at low enzyme levels [8]. The tambaqui proteases were effective, that is, they retained their activities and were able to degrade proteins even at lower concentration of 0.2 mg.mL^{-1} and in the presence of detergent solutions.

The high cost of the purification protocols has been a limiting factor to technical utilization of fish proteases [11,13]. Ethanol precipitation is a technique that can be applied to proteins on an industrial scale, owing to its good physicochemical properties, such as complete miscibility with water, good freezing-point depression, absence of explosive mixtures, high volatility, chemical inertness, low toxicity and low cost, especially in Brazil [23,39-41]. Moreover, this method was efficient to remove the fish viscera smell characteristic and to clarify the crude extract. The ethanol precipitation also showed to be a useful, rapid and low cost protocol to obtain a proteolytic extract with potential use as a laundry detergent additive, from fish processing waste. Thus, the alkaline proteases from *C.*

macropomum intestine have maximum activity at high pH and temperature range that indicates its usefulness in a wide range of temperature washing machine programmers.

4. Conclusions

Ethanol precipitation protocol was carried out to obtain proteases (trypsin-like enzyme mostly) from *C. macropomum* intestines. These proteases acted best inside the pH and the temperature recommended for enzymes as laundry additives. Also, they showed to be stable at these pH and temperature. They were also stable in the presence of non-ionic and ionic surfactants, except SDS. Furthermore, they were stable at high H_2O_2 concentrations and in the presence of several commercial detergents (Ala®, Bem-te-vi®, Omo® and Surf®). The application of these proteases as laundry additive has the following advantages: simple and low cost obtaining protocol; fish processing waste as the source of the proteases, adding value to this industrial by-products and compatibility characteristics with detergent formulations.

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Table 1. Partial purification of proteases from C*olossoma macropomum* intestine by ethanol precipitation. Protein and enzymatic activity were established, respectively, according to Warburg and Christian [24] and Alencar et al. [12] using 1% (w/w) azocasein as substrate.

Samples	**Total activity (U)**	**Total protein (mg)**	**Specific activity (U/mg of P)**	**Purity (fold)**	**Yield (%)**
Crude extract	46,875	834	56.2	1.0	100.0
Heated crude extract	44,574	623	71.6	1.3	95.1
F1: Ethanol 0-30%	6,971	448	15.6	0.3	14.9
F2: Ethanol 30-70%	35,114	481	73.0	1.3	74.9
Final supernatant	1,921	97	19.8	0.4	4.1

Table 2. Effect of inhibitors on proteases of *Colossoma macropomum* intestine purified by ethanol precipitation. Enzymatic activity was assayed at 25ºC and pH 8.0 as described in section 2.5.

Substrate	**Enzymatic Activity**	**Inhibition (%)**			
	mU/mL	**Benzamidine**	**TLCK**	**PMSF**	**TPCK**
BapNA	1.59	100	98	54	
Suc-Phe-p-Nan	0.04			96	100

BApNA: benzoil arginine p-nitroanilyde; Suc-Phe-p-Nan: succinyl phenylalanine p-nitroanilyde; PMSF: phenylmethylsulfonylfluoride; TPCK: tosyl phenylalanine chloromthyl ketone; TLCK: tosyl lysine chloromethyl ketone

Table 3. Effect of surfactants on proteases of *Colossoma macropomum* intestine purified by ethanol precipitation. Preparation was incubated with the surfactants below, samples were withdrawn at the indicated time interval and their residual activities established at 25^{o}C, pH 8.0, using azocasein as substrate, as described in Section 2.8.

Surfactants (1% w/v)	**Residual activity* %**	
	After 30 min	**After 60 min**
Saponin	117.5 ± 0.3	118.4 ± 2.1
Sodium choleate	94.2 ± 7.3	107.3 ± 4.4
Tween 20	117.3 ± 5.4	108.2 ± 0.5
Tween 80	112.0 ± 8.1	107.8 ± 4.7
SDS	15.1 ± 1.3	7.3 ± 1.0

* Values are expressed in ± standard deviation. n =4.

Figure legends

Fig. 1. SDS-PAGE of alkaline protease from *Colossoma macropomum* viscera. Lane 1: molecular weights standard protein markers of 66, 46, 36, 29, 24 and 14.2 kDa; Lane 2: crude extract; Lane 3: precipitation with ethanol 30-70% and zimogram, Lane 4: crude extract; Lane 5: precipitation with ethanol 30-70%.

Fig. 2. Effect of pH on the activity of proteases precipitated by ethanol from *Colossoma macropomum* intestine. The enzyme activity on azocasein was established at different pH provided by the following buffer solutions: 0.1M phosphate (■), Tris-HCl (●) and NaOH/glycine (▲).

Fig. 3. Alkaline stability of proteases precipitated from *Colossoma macropomum* intestine. The enzyme was previously incubated in 0.1 M NaOH/glycine buffer at the indicated pH for 30 min and afterwards its activity was determined in 0.1 M Tris-HCl, pH 11.0.

Fig. 4. Temperature profile (a) and thermal stability (b) proteases from *Colossoma macropomum* intestine precipitated by ethanol. a) The protease activity was assayed at indicated temperatures, pH 8.0, and b) the enzyme preparation was incubated for 30 min at the indicated temperatures and after preparation reached 25ºC their proteolytic activities were assayed.

Fig. 5. Inactivation curve by H_2O_2 of protease from *Colossoma macropomum* intestine precipitated by ethanol. Enzyme preparations were incubated at 40º C with H_2O_2 at the concentrations of 5% (●); 10% (▲); 15% (▼). Samples were withdrawn at time interval their activities (duplicates) established using azocasein as substrate and compared to the non-treated sample (■).

Fig. 6. Stability of protease in commercially available detergents. Protease (0.2 mg mL^{-1}) was incubated at 40ºC in presence of detergents at 7mg mL^{-1}. Activity of control sample devoid of any detergent incubated under similar conditions (●), Surf® (□); Ala® (△);. Bem-te-vi® (▼); Omo Multi-Ação® (◇).

Fig.1

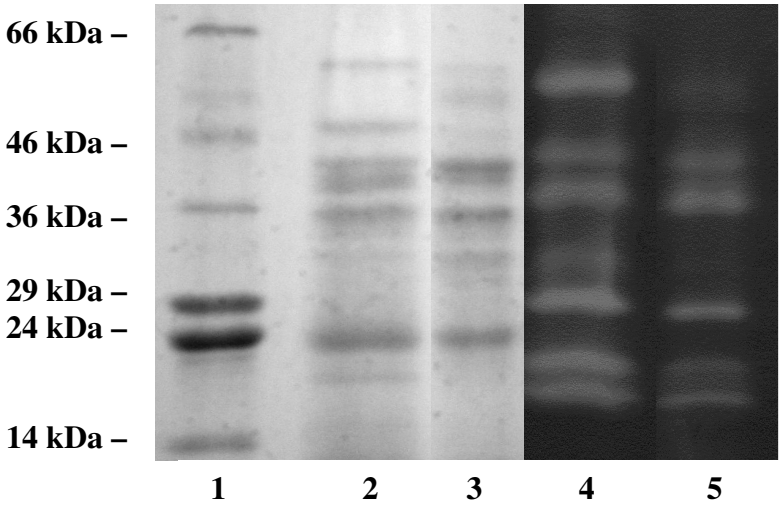


Fig. 2

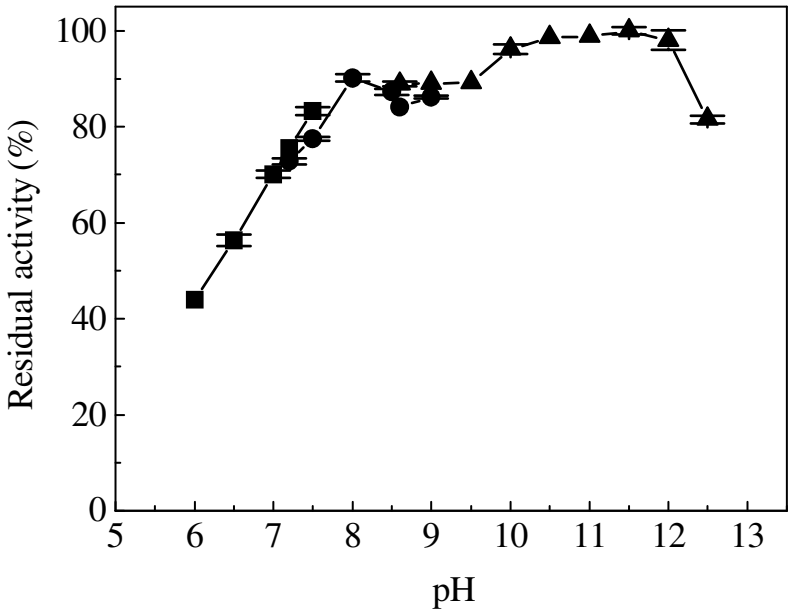

Residual activity (%)
100
80
60
40
20
0
5
6
7
8
9
10
11
12
13
pH

Fig. 3

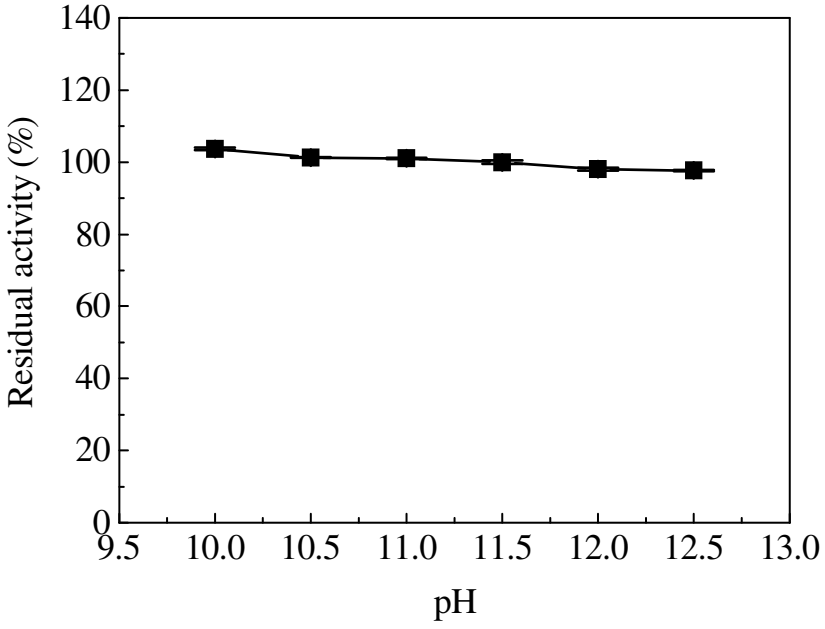
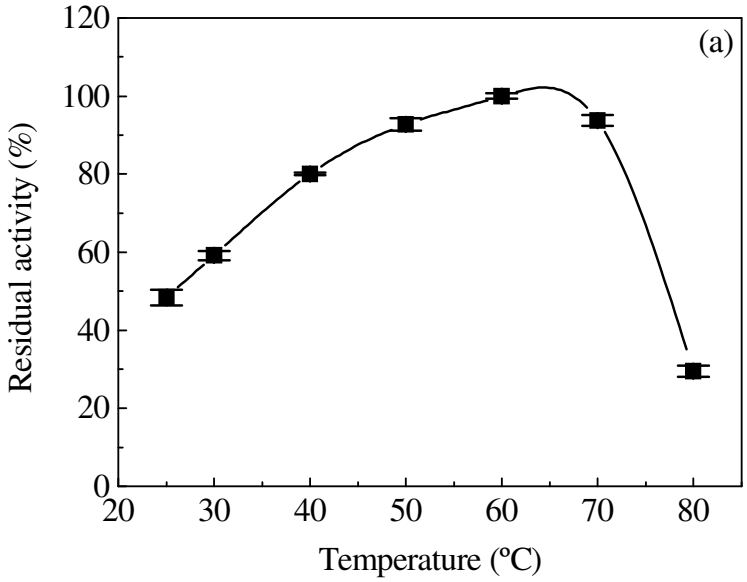

Residual activity (%)
140
120
100
80
60
40
20
0
9.5
10.0
10.5
11.0
11.5
12.0
12.5
13.0
pH

Fig. 4


(a)
Residual activity (%)
Temperature (°C)
0
20
40
60
80
100
120
20
30
40
50
60
70
80

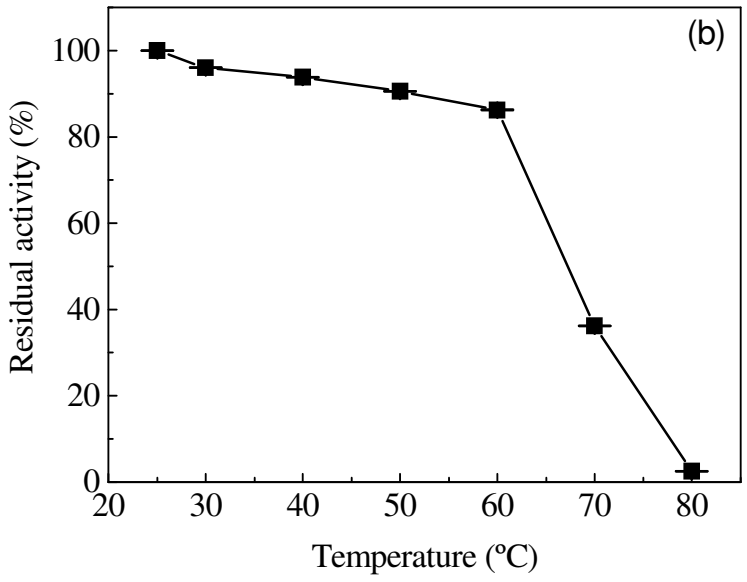

(b)
Residual activity (%)
Temperature (°C)
0
20
40
60
80
100
20
30
40
50
60
70
80

Fig. 5

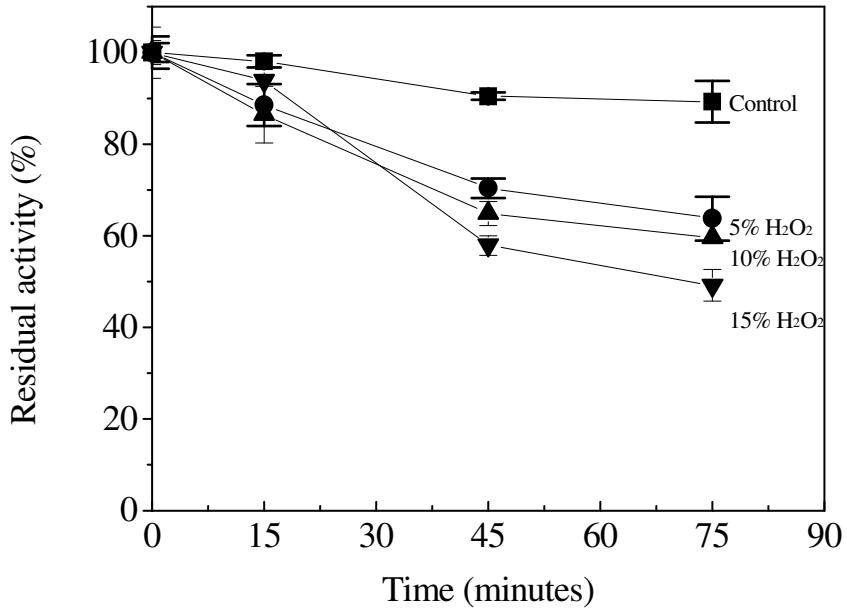
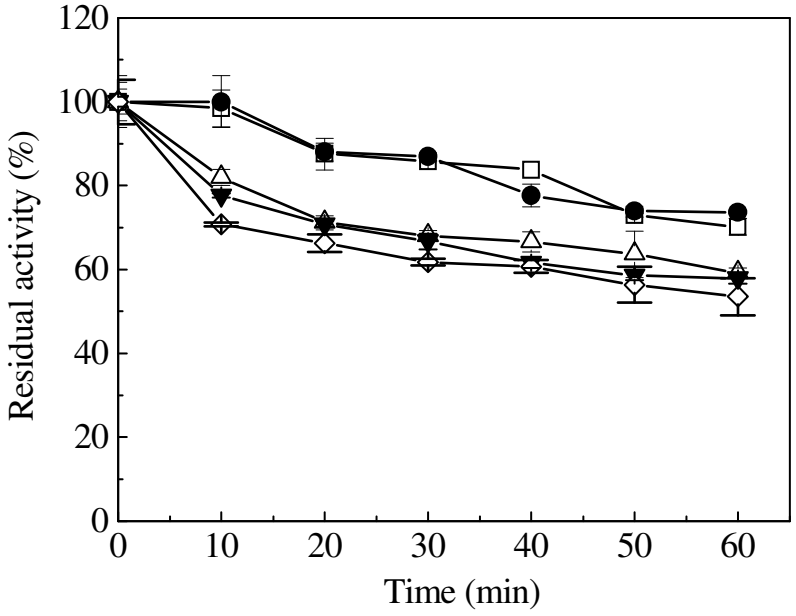

Residual activity (%)
Time (minutes)
Control
5% H2O2
10% H2O2
15% H2O2
0
20
40
60
80
100
0
15
30
45
60
75
90

Fig. 6


Residual activity (%)
Time (min)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

Effect of surfactants and oxidants on proteases from the freshwater fish common carp (*Cyprinus carpio* L)

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Abstract

Alkaline proteases are commercially important enzymes due to its broad range applications. Intestines from *Cyprinus carpio* L., which is an important fish in the Brazilian aquaculture, present a high amount of proteases are proposed in this work as a source of such molecules. The crude extract was submitted to a partial purification by ethanol precipitation. The fraction 30-70% ethanol presented the highest proteolytic activity and was submitted to further studies. The proteolytic activity of this was assayed at different temperatures and pH. Temperature and pH stability experiments were also carried out. Different concentrations of commercially available detergents, surfactants and hydrogen peroxide were used to test the compatibility of these proteases. The fraction containing 30-70% of ethanol saturation showed higher recovery (142.4%) and specific activity (67.4 U/mg protein) when compared to the crude extract. The optimal temperature and pH were found to be 50ºC and 11.0, respectively. The proteases were stable in a pH range of 6.5 – 12.0. The enzymatic activity was strongly activated by non-ionic commercially available surfactants (Tween 20 and Tween 80) and retained more than 80% and 60% of their initial proteolytic activity at 40ºC for 1 hour in the presence of the ionic surfactants sodium cholate and saponin, respectively. In addition to that almost 50% of enzymatic activity was retained in the presence of 5% (v/v) of hydrogen peroxide at 40ºC after 75 min of incubation. The high proteolytic activity of the proteases at extreme temperatures and alkaline pH together with its stability in the presence of the surfactants and oxidants tested indicate that these alkaline proteases can well be used in detergent formulations.

Key-words: Alkaline proteases, *Cyprinus carpio*, surfactants, oxidants, termostable.

1. Introduction

Proteases are responsible for a large variety of functions in biological systems and have important biotechnological applications. Proteases represent one of the three largest groups of industrial enzymes and find application in detergent, leather, food, and pharmaceutical industries and bioremediation processes (Anwar and Salemuddin, 1998; Gupta et al., 2002). Proteases are one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures (Rao et al., 1998). Among the various proteases available in the market, bacterial proteases are the most commercially used when compared to animal and fungal proteases (Ward, 1985). The protease extracted from members of the genus *Bacillus* account for the highest amount of protease produced at present. Although subtilisins have been the enzymes of choice for detergent formulations they are not the ideal detergent enzymes (Samal et al., 1990). Thus it is desirable to search for new proteases with novel properties from as many different sources as possible (Banerjee et al., 1999).

Fish viscera generated during the processing is a potential source of enzyme, namely proteinases, which may have some unique properties for industrial application (Ooshiro, 1971; Haard, 1992; Klomklao et al., 2005). Those viscera accounts for 5% of total fish mass and could be well transformed by relatively simple procedures into valuable products such as enzymes (Castillo-Yánez et al., 2005).

Alkaline proteases from various fishes have been characterizated showing desirable properties for its use in industrial processes (Cohen et al., 1981; Jónás et al., 1983; Guizani et al., 1991; El-Shemy and Levin, 1997; Aranishi et al., 1998; Bezerra et al., 2000; Bezerra et al., 2001; Alencar et al., 2003; Bezerra et al., 2005; Castillo-Yánez et al., 2005; Souza et al.,

2006). However, there is no available information in the literature regarding fish proteases and their compatibility with surfactants and oxidants.

The common carp is a temperate fish that tolerates temperatures ranging from 3 to 32ºC. These fishes are omnivorous and stomachless. It is the most important fish of the aquaculture in South Brazil and is responsible for more than 50% of fish production in 2004 in that region (IBAMA, 2005).

Aranishi et al. (1998) have previously reported that dipeptidases extracted and purified from common carp displayed desirable characteristics for its use in biotechnology processes. Cohen et al. (1981) and Jónás et al. (1983) have already foreseen the potential of proteolytic enzymes extracted from this fish in the early eighties.

The present investigation reports the partial purification of *Cyprinus carpio* viscera and aims to test the effect of temperature, surfactants and oxidants in alkaline protease obtained from common carp.

2. Material and methods

2.1 Enzyme extraction

Intestines of *Cyprinus carpio* were collected and homogenized (20 mg of tissue/mL of 0.01 M Tris-HCl pH 8.0) by using a tissue homogenizer. The resulting preparation was centrifuged at 10,000 x g for 10 min at 10ºC to remove cell debris and nuclei. The supernatant (crude extract) was used for purification steps (adapted from Bezerra et al., 2005).

2.2 Enzyme partial purification

The crude extract was incubated at 45ºC for 30 min and centrifuged at 10,000 x g for 10 min at 4ºC (Bezerra et al., 2001). Protein precipitation by ethanol was performed according

to Cortez and Pessoa Jr. (1999) with slight modifications: the supernatant was fractionated with iced ethanol slowly added under mild stirring at 4ºC to obtain fractions of saturation from 0-30% (F_1) and from 30-70% (F_2). After the complete addition of ethanol the mixture was left to rest during 1 hour and centrifuged at 10,000 x g at 4ºC for 15 min. The pellet was dissolved using cold 0.1 M Tris-HCl buffer (pH 8.0). The samples were dialyzed against 0.05 M Tris-HCl buffer pH 8.0.

2.3 Enzyme assay

Alkaline proteases activities were assayed at 25ºC as described by Alencar et al. (2003). Azocasein 1.0% (w/v) in 0.1 M Tris-HCl buffer was used as substrate. One unit (U) of enzyme activity was defined as the amount of enzyme capable to hydrolyze azocasein giving an increase of 0.001 units of absorbance per minute. The total protein content was determined in duplicate by Warburg and Christian (1941).

2.4 Effect of pH

Protease activity was measured at different pH values under standard assay conditions using azocasein as substrate. The enzymatic activity was assayed using 0.1 M phosphate buffer (pH 6.5 – 7.5), 0.1 M Tris-HCl buffer (pH 7.2 – 9.0) and 0.1 M NaOH/Glycine buffer (pH 8.6 - 12.5). The effect of pH on enzyme stability was studied by incubating the enzyme at 25ºC with buffers of different pH values from 6.5 – 12.5 (0.1 M buffer as described above) and then proteolytic activity was measured at 25ºC using 0.1 M Tris-HCl buffer at optimum pH.

2.5 Effect of temperature

The proteolytic activity was assayed at various temperatures (25 – 80ºC) to determine the optimum temperature at pH 8.0. For determination of thermal stability, previously to enzymatic activity the enzyme preparation was incubated for 30 min at the temperature tested and after the temperature had reached 25ºC the proteolytic activity was assayed.

2.6 Effect of oxidizing agent and surfactants

The enzyme was incubated at 5 and 10% concentrations of hydrogen peroxide at 40º C for 15, 40 and 75 min and enzyme stability was determined in terms of residual proteolytic activity. Solutions of ionic (saponin and sodium cholate) and non-ionic surfactants (tween 20 and tween 80) were tested at concentrations of 1% (w/v) for 30 and 60 min at 40ºC after which the enzyme activity was assayed (Moreira et al., 2002).

2.7 Compatibility with commercial detergents

The protease at a concentration of 0.20 mg ml^{-1} was incubated at 40ºC with commercially available detergents: Ala® (Protec & Gamble); Bem-te-vi® (Alimonda); Omo Multi Ação® (UniLever) and Surf® (UniLever) to a final concentration of 7 mg ml^{-1}. Samples (150 µl) were removed after a time interval of 10 min (total period of 60 min). The residual proteolytic activity in each sample was determined at 25ºC, assayed and compared with the control sample incubated at 40ºC without detergent (Moreira et al., 2002).

3. Results and discussion

Heat treatment has proven to be an important strategy in fish protease purification because it denatures and removes distinct heat-labile proteins in the crude extract as reported by Bezerra et al. (2001, 2005). Cortez and Pessoa Jr. (1999) reported that ethanol, used in this work as the precipitant agent, is by far the most important of the solvents in protein precipitation, owing to its good physicochemical properties, namely complete miscibility with water, good freezing-point depression, absence of explosive mixtures, high volatility, chemical inertness, low toxicity and low cost, especially in Brazil.

Thermostable dipeptidases were purified by Aranish et al. (1998) from carp intestine, increase in specific activity of 372 fold and 2% recovery . This work had the distinct aim of studying the digestion of dietary protein and the absorption of free aminoacids in carp intestine. But the proteases used in the food and detergent industries are prepared in bulk quantities and used as crude preparations (Rao et al., 1998). In our work the extract was purified 2.5 fold and 142.4% recovery (Table 1). Therefore, in this specific case, partial purification using ethanol as a precipitant agent was an efficient process to remove the viscera characteristic smelt and to clarify the crude extract.

The carp intestine enzyme seems to be a typical alkaline enzyme on account of its remarkable action at high pH (Figure 1). The optimum pH of these enzymes (11.0) was higher than the results reported by Aranish et al. (1998) and Jónás et al. (1983), 9.0 and 10.0, respectively, for carp proteases. Otherwise, in this study the carp proteases exhibited optimum hydrolysis at 50ºC and were stable up to this temperature. The up cited authors found higher stability (70ºC) (Aranish et al., 1998) and (55ºC) for proteolytic enzymes from intestine as reported by Jónás et al. (1983). However, in this last study the protease lost 50% of its initial activity after 1.3 min at optimum temperature. On the other hand, our results showed that the

proteins under study retained 100% of its initial activity after 30 min of incubation at 50ºC (Figure 2). It also indicates that carp intestinal proteases is appropriately defined as a thermostable enzymes, similarly to proteases from intestine of Nile tilapia (*Oreochromis niloticus*) (Bezerra et al., 2005) and mullet (*Mugil cephalux*) (Guizani et al., 1991).

A good detergent protease shoul be stable in the presence of surfactants and oxidants agents. In the presente case, proteases from *Cyprinus carpio* are almost stable or stimulated in presence of ionic and non-ionic surfactants. The maximums stimulations were with 1% Tween 20 and Tween 80 where the activity increased 126.5% and 119.5% after 60 min of incubation at 40ºC, respectively. In the presence of 1% ionic surfactants, Sodium cholate and Saponin, the carp proteases retained 86.2% and 64%, respectively, of its activity after incubation as describe above (Table 2). These results are higher than that found by Moreira et al. (2002) for protease from fungus *Nocardiopsis* sp.

A good number of bacterial alkaline proteases are commercially available, such as Subtilisin Carlsberg, Subtilisin BPN' and Savinase with their major application as detergent enzyme. However, not all of them are stable or active in presence of bleaching/oxidizing chemicals (Table 3). Hence, the latest trend in enzyme-based detergents is the use of rDNA and protein engineering technologies to produce bioengineered enzymes with better stability. Mutations have led to newer protease preparations with improved catalytic efficiency and better stability towards temperature, oxidizing agents and changing wash conditions (Gupta et al., 2002, Rao et al., 1998).

The peroxide inactivation curve (Figure 3), shows that the proteases are stable even at high concentrations of H_2O_2 (10% v/v) per 45 min with 60% residual activity. The oxidation-stability in most of the commercial proteases, Durazym, Maxapem and Purafect (Table 3) has been introduced through site directed mutagenesis and protein engineering techniques (Gupta et al., 2002, Rao et al., 1998). However, the present *Cyprinus carpio* intestinal proteases have

an advantage over the existing commercial proteases, as appreciable oxidation stability is already present in this enzyme.

As seen from Figure 4, at least 50% of the activity was lost after incubation for only 10 min at 40ºC in the presence of all available laundry detergents. These results suggested that alkaline protease from *Cyprinus carpio* are inhibited to laundry detergents components. The detergents contain high amounts of chelating agents, used as water softeners and to also assist in stain removal. These agents specifically bind to and chelate metal ions making them unavailable in the detergent solution (Beg and Gupta, 2003). It is can indicate that the proteases from carp require metal cofator, but this conclusion can only be confirmed after further study with EDTA.

The high proteolytic activity of the proteases at pH 11.0 and 50ºC and stability in the presence of various surfactants and high concentration of oxidants suggest its suitability for inclusion in detergent compositions, with the exception of laundry detergents, whose presence inhibited the proteases activity studied. Therefore, the author suggest test with other kinds of detergents like those used for cleaning contact lenses or dentadures.

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Table 1

Partial purification of proteases from *Cyprinus carpio* intestine. Proteins and enzymatic activities were established, respectively, according to Warburg and Christian (1941) and Alencar et al. (2003) using azocasein (1% w/v) as substrate.

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purity (fold)	Yield (%)
Crude extract	4,777.0	178.5	26.8	1.0	100.0
Heated Crude Extract	4,065.3	87.1	46.7	1.7	85.1
F1: Ethanol 0-30%	738.1	65.2	11.3	0.4	15.5
F2: Ethanol 30-70%	6,801.2	101.0	67.4	2.5	142.4
Final supernatant	157.6	33.8	4.7	0.2	3.3

Table 2

Effect of surfactants on proteolitic activity of *Cyprinus carpio* intestine purified by ethanol precipitation. The preparation was incubated wih the surfactants below, samples were withdrawn at the indicated time interval and their residual activities established at 25ºC, pH 8,0, using azocasein as substrate, as described in Section 2.6

Surfactant (1% w/v)	Residual Activity (%)	
	After 30 min	After 60 min
Twen 20	125 ±1.5	126.5±6.7
Tween 80	117.4±3.3	119.5±0
Sodium choleate	78.6±1.2	86.2±11.2
Saponin	59.9±1.5	64±4.2

* Values are expressed in ± standard deviation (duplicates).

Table 3.Comparation of pH, temperature and bleach stability properties of commercial detergent proteases with alkaline protease from *Cyprinus carpio*

Commercial proteases	Sources	pH optima/ range	Temperature optima (ºC)/range	Bleach/oxidant stability	Suppliers
This work	*Cyprinus carpio*	11.0	50	+	-
Alcalase	*Bacillus licheniformis*	7-10	60	-	Novozymes, Denmark
Savinase	*Bacillus lentus*	8-11	50-60	-	Novozymes, Denmark
Esperase	*Bacillus lentus*	7-12	50-65	-	Novozymes, Denmark
Maxatase	*Bacillus subtilis*	9-10	50-60	-	Gist-brocades, The Netherlands
Opticlean	*Bacillus alcalophilus*	8-11.5	15-60	-	Solvay enzymes GmbH, Germany
Optimase	*Bacillus licheniformis*	7.5-10.5	15-65	-	Solvay enzymes GmbH, Germany
Everlase	Protein engineered variant of Savinase®	8-11	50	+	Novozymes, Denmark
Durazym	Protein engineered variant of Savinase®	8-11	50	+	Novozymes, Denmark
Maxapem	Protein engineered variant of *Bacillus* sp.	11-12	60	+	Solvay enzymes GmbH, Germany
Purafect	Genetic engineered donor *Bacillus lentus* expressed in *Bacillus* sp.	10	40-65	+	Genecor International, Inc., USA

Figure legends

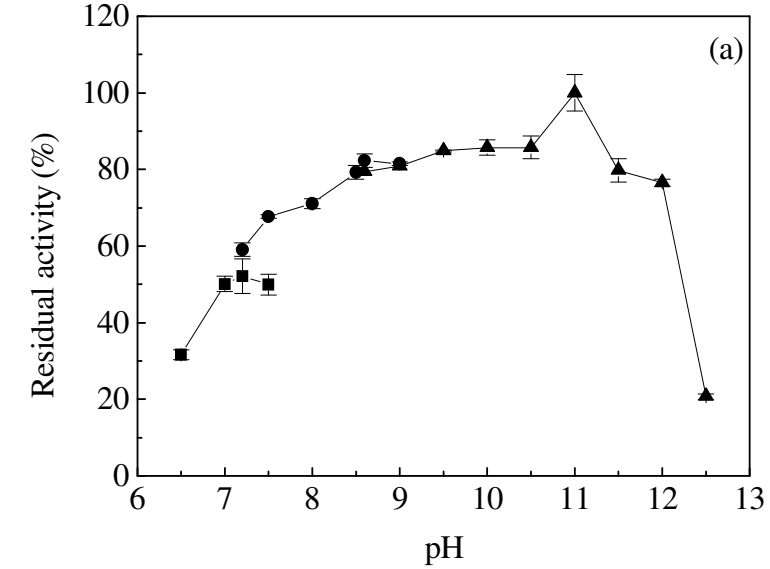
Fig. 1. Effect of pH on proteolytic activity (a) and on stability (b) of protease . Buffer solutions 0.1M phosphate (■); Tris-HCl (●) and NaOH/glycine (▲) of *Cyprinus carpio* viscera 30-70% ethanol fraction.

Fig. 2. Temperature profile (a) and thermal stability (b) of *Cyprinus carpio* viscera 30-70% ethanol fraction.

Fig. 3. Effect of peroxide on activity of proteases from *Cyprinus carpio* alimentary canal 30-70% ethanol fraction at 40ºC.

Fig. 4. Stability of protease in commercially available detergents. Protease (0.2 mg ml^{-1}) was incubated at 40ºC in presence of detergents at 7mg ml^{-1}. Activity of control sample devoid of any detergent incubated under similar conditions (■), Ala® (●), Bem-te-vi® (▲), Surf® (▼) and Omo Multi-Ação® (◆).

Fig. 1


(a)
Residual activity (%)
pH
0
20
40
60
80
100
120
6
7
8
9
10
11
12
13

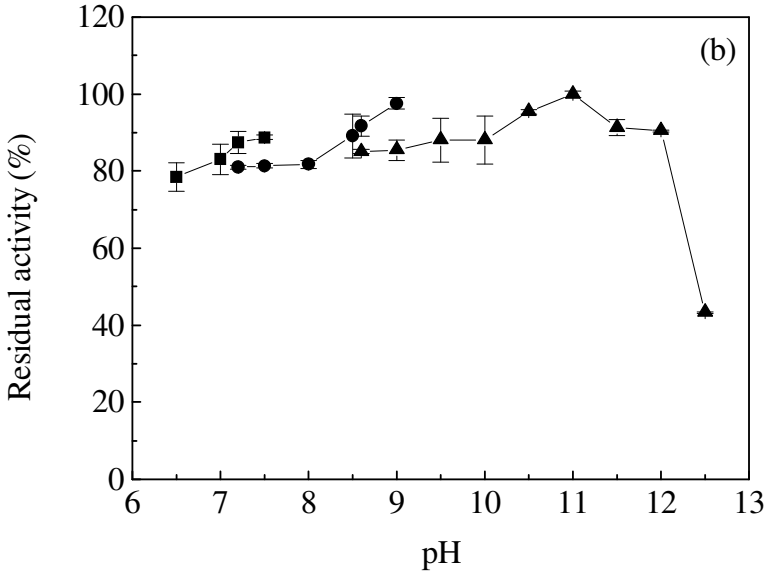

(b)
Residual activity (%)
pH
0
20
40
60
80
100
120
6
7
8
9
10
11
12
13

Fig. 2

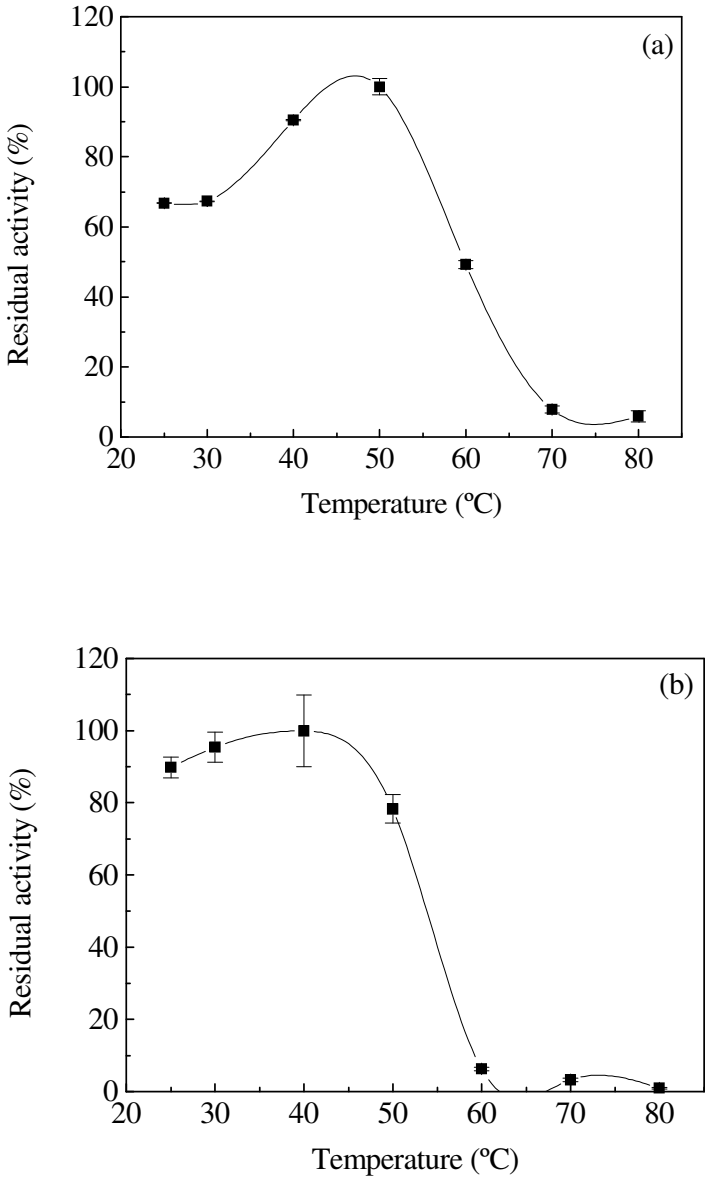

(a)
Residual activity (%)
Temperature (ºC)
120
100
80
60
40
20
0
20
30
40
50
60
70
80
(b)
Residual activity (%)
Temperature (ºC)
120
100
80
60
40
20
0
20
30
40
50
60
70
80

Fig.3

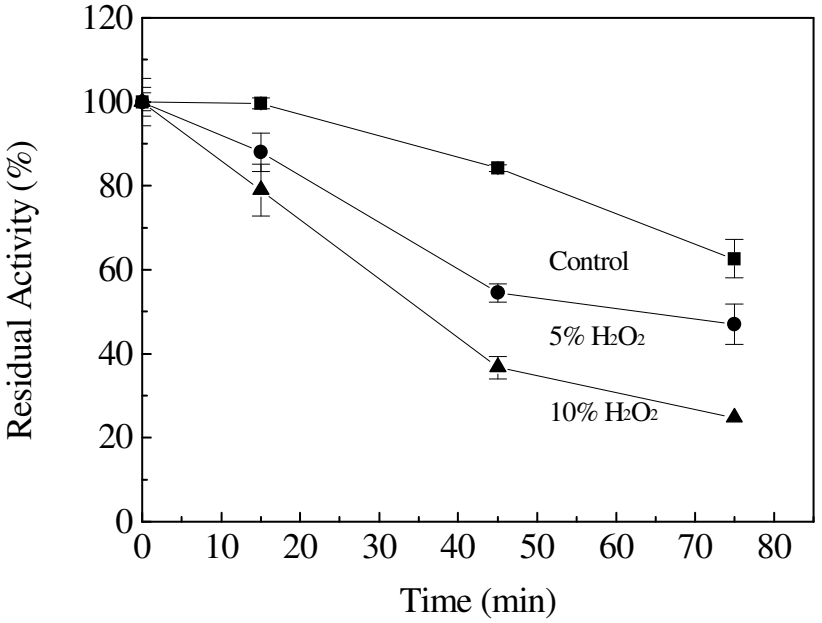
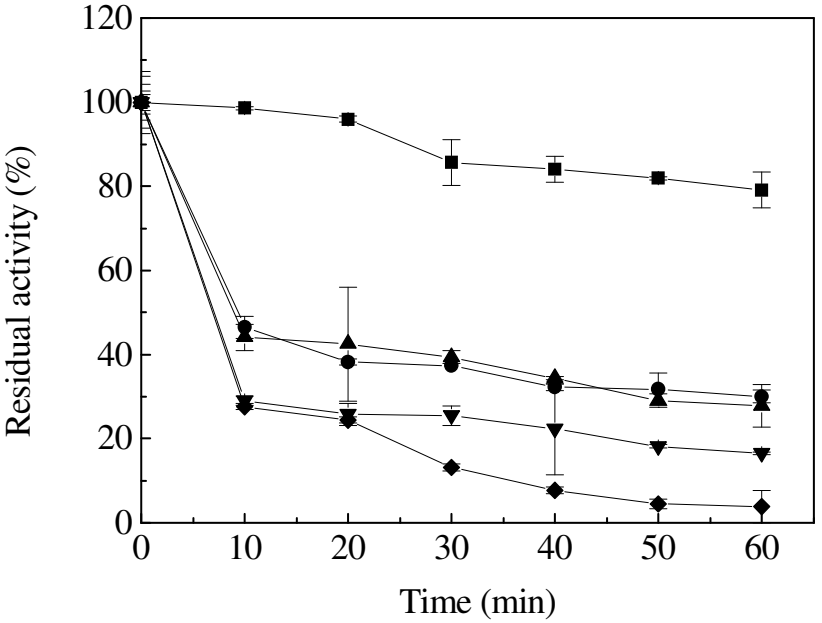

Residual Activity (%)
Time (min)
Control
5% H2O2
10% H2O2

Fig.4


Residual activity (%)
Time (min)

5 CONCLUSÕES

1. Proteases são parcialmente purificadas com tratamento térmico e precipitação com álcool etílico na fração de saturação de 30 a 70% ($F_{30-70\%}$). Constituindo um método barato, não corrosivo para a indústria e com baixo nível de poluição ambiental.
2. Proteases parcialmente purificadas na $F_{30-70\%}$ apresentam rendimentos e atividade superiores às outras frações.
3. As proteases parcialmente purificadas na $F_{30-70\%}$ são alcalinas (pH 10,0-12) e apresentam temperaturas ótimas elevadas (50ºC e 60 ºC).
4. As proteases da carpa são estáveis até 50°C e em uma faixa de pH que varia de 6,5-12,0, enquanto as do tambaqui foram termoestáveis até 60°C e estáveis em uma faixa de pH entre 6,0-12,5, por 30 minutos.
5. Proteases encontradas na $F_{30-70\%}$ do tambaqui podem ser usadas como aditivos nos detergentes comerciais Surf®, Ala®, Bem-te-vi® e OMO® por terem sido estáveis por 1 hora em condições de lavagem e na presença de diferentes agentes surfactantes e oxidantes.
6. As proteases da $F_{30-70\%}$ da carpa apesar de não terem sido estáveis na presença de diferentes detergentes comerciais, apresentaram estabilidade na presença de diferentes agentes surfactantes (iônicos e não-iônicos) e na presença de agentes oxidantes, sugerindo que alguns ingredientes dos detergentes em pó comerciais inibe a sua atividade. No entanto, essa constatação não descarta o uso dessas proteases como um aditivo na indústria de detergentes, podendo ser utilizadas, por exemplo, como aditivos nos detergentes para lentes de contato e dentaduras.

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Anexo A

Guide for Authors: Process Biochemistry

Process Biochemistry is an application-orientated research journal devoted to reporting advances with originality and novelty, in the science and technology of the processes involving bioactive molecules or elements, and living organisms ("Cell factory" concept). These processes concern the production of useful metabolites or materials, or the removal of toxic compounds. Within the segment "from the raw material(s) to the product(s)", it integrates tools and methods of current biology and engineering. Its main areas of interest are the food, drink, healthcare, energy and environmental industries and their underlying biological and engineering principles. Main topics covered include, with most of possible aspects and domains of application: fermentation, biochemical and bioreactor engineering; biotechnology processes and their life science aspects; biocatalysis, enzyme engineering and biotransformation; downstream processing; modeling, optimization and control techniques.

Submission of manuscripts

Authors are requested to submit their manuscripts electronically, by using the EES online submission tool at http://ees.elsevier.com/prbi/. After registration, authors will be asked to upload their article, an extra copy of the abstract, and associated artwork. The submission tool will generate a PDF file to be used for the reviewing process. The submission tool generates an automatic reply and a manuscript number will be generated for future correspondence. A cover letter should be submitted on line by authors together with the manuscript, which includes the following points: 1) all authors agree to submit the work to PRBI, 2) the work has not been published/submitted or being submitted to another journal, 3) the novelty and significant contribution of the submitted work are briefly described, 4) the transfer of copyright from the author to the publisher.

In their on-line submission, authors are required to suggest at least two independent referees (up to five, outside their own institution) with their email addresses. But, the selection of the referees is up to the Editors. All submissions will be reviewed by two referees.

Format and type of manuscripts

Process Biochemistry accepts three types of manuscripts: Full length articles, Short communications and Reviews. The text must be as concise as possible. All manuscripts must follow the following presentation style: the text with double-space, the reference list, a separate page of figure legends, and finally tables and figures with a separate page for each one. It is highly recommended that the legends to be as complete and concise as possible: one figure or one table should be perfectly understandable with its own legend. Incomplete legends could not be accepted.

Full length articles (FLA) should not generally exceed 25 double-spaced pages of text (not including the references) and should not contain more than 15 figures and/or tables. Each paper should be provided with an abstract of 100-150 words reporting concisely on the purposes and results of the paper, and also six keywords.The title of the paper should unambiguously reflect its contents. Where the title exceeds 70 characters a suggestion for an abbreviated running title should be given.

The SI system should be used for all scientific and laboratory data: if, in certain instances, it is necessary to quote other units, these should be added in parentheses. Temperatures should be given in degrees Celsius. The unit 'billion' (109 in America, 1012 in Europe) is ambiguous and should not be used. Abbreviations for units should follow the suggestions of the British Standards publication BS 1991. The full stop should not be included in abbreviations, e.g. m (not m.), ppm (not p.p.m.), % and / should be used in preference to 'per cent' and 'per'. Where abbreviations are likely to cause ambiguity or may not be readily understood by an international readership, units should be put in full. Footnotes should be avoided especially if they contain information which could equally well be included in the text. The use of proprietary names should be avoided. Papers essentially of an advertising nature will not be accepted.

References

References should be cited at the appropriate point in the text by a number in square brackets. A list of references, in numerical order, should appear at the end of the paper. All references in this list should be indicated at some point in the text and vice versa. Unpublished data or private communications should not appear in the list. Examples of layout of references are given below:

1. Treshow, M.,Environment and Plant Response. McGraw-Hill, New York, 1970.

2. Chang, C.W., Fluorides. In Responses of Plants to Air Pollution, ed. J.B. Mudd and T.T. Kozlowski. Academic Press, New York, 1975, pp. 57-95.

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Anexo B

Guide for Authors:Journal of Biotechnology

Journal of Biotechnology provides a medium for the rapid publication of both full-length articles and short communications on all aspects of biotechnology. The Journal will accept papers ranging from genetic or molecular biological aspects to those covering biochemical, chemical or bioprocess engineering aspects, provided that in each case the material is directly relevant to biotechnological systems. Papers presenting information of a multi-disciplinary nature, that would not be suitable for publication in a journal devoted to a single discipline, are particularly welcome. The following areas are covered by the Journal: Nucleic Acids / Molecular Biology; Physiology / Biochemistry; Biochemical Engineering / Bioprocess Engineering; Industrial Processes / New Products; Medical Biotechnology.

Submission of manuscripts

Submission of a paper to the Journal of Biotechnology implies: (1) that it is not being submitted for publication elsewhere; (2) the transfer of the copyright from the author to the Publisher.

Authors are requested to submit their manuscripts electronically, by using the EES online submission tool at http://ees.elsevier.com/jbiotec/. After registration, authors will be asked to upload their article, an extra copy of the abstract, and associated artwork. The submission tool will generate a PDF file to be used for the reviewing process. The submission tool generates an automatic reply and a manuscript number will be generated for future correspondence.
Authors in Asia please note that upon request, Elsevier Japan will provide authors with a list of people who can check and improve the English of their paper (before submission). Please contact our Tokyo office: Elsevier Science Japan, 9-15 Higashi-Azabu 1-chome, Minato-ku, Tokyo 106-0044; Tel. +81- (0)3-3589 5019; Fax +81-(0)3-3589-5044. Authors should further note that final acceptance resides with the Chief Editor, Professor A. Puhler.

Types of papers

(1) Full-length papers, generally not exceeding 20 typewritten pages. Full-length papers should:(a) be divided into sections (Abstract, Introduction, Materials and methods, Results, Discussion);(b) contain an Abstract, not exceeding 200 words, at the beginning of the paper, followed by 3-6 keywords; (c) not exceed 12-15 printed pages (approximately 20 typewritten pages) including the space required for figures. Longer papers will be considered, but may be subject to delayed publication.

Manuscripts

The manuscript should be typed with double spacing and wide margins, and should be accompanied by a separate title page giving the authors' names and affiliations, as well as an address for correspondence including fax number and e-mail address. If it is a resubmission this has to be indicated and the former J. Biotechnol. MS No. has to be given. All pages have to be numbered consecutively, including the abstract, figure legends, and tables. Place the last two items after the References section. Copies of in-press and submitted manuscripts that are important for judgement of the present manuscript should be enclosed to facilitate reviewing.
For the title, avoid numbered series titles.
In the Abstract, avoid abbreviations and references. The Abstract should be followed by up to six Keywords.
The Material and Methods section should include sufficient technical information to allow the experiments to
The Material and Methods section should include sufficient technical information to allow the experiments to be repeated. When experimental conditions are critical, give enough information to enable another investigator to repeat the procedure. For commonly used methods a simple reference is sufficient. If several alternative methods are described in the paper cited, please identify the method briefly in addition to the reference. Describe new methods completely.
Present the Results as concise as possible in either table(s) or figure(s). Avoid extensive use of graphs to present data that might be more concisely presented in the text or tables. The Results and Discussion sections may be combined.
References should be assembled on a separate sheet. In the text they should be referred to by name and year (Harvard System). More than one paper from the same author in the same year

must be identified by the letters a, b, c, etc., placed after the year of publication. In the text, when referring to a work by more than two authors, the name of the first author should be given followed by et al. and year in brackets. Literature references must consist of names and initials of all authors, year, title of paper referred to, abbreviated title of periodical, volume number and first and last page numbers of the paper. Periodicals, books and multi-author books should follow the examples below:

Ponti, C., Sonnleitner, B. and Fiechter, A. (1995) Aerobic thermophilic treatment of sewage sludge at pilot plant scale. 1. Operating conditions. J. Biotechnol. 38, 173-182. Walter, H., Brooks, D.E. and Fisher, D. (1985) Partitioning in aqueous two-phase systems. Academic Press, Inc., Orlando, FL. Hamer, G. (1989) Fundamental aspects of aerobic thermophilic digestion. In: Bruce, A.M., Colin, F. and Newman, P.J. (Eds.), Treatment of Sewage Sludge: Thermophilic Aerobic Digestion and Processing Requirements for Landfilling. Elsevier Applied Science, London, pp. 2-19.

Abbreviations of journal titles should conform to those adopted by List of Serial Title Word Abbreviations, International Serials Data System, 20 rue Bachaumont, 75002 Paris, France. ISBN 2- 904938-02-8.

Tables should be typed double-spaced on separate sheets, numbered consecutively with Arabic numerals, and only contain horizontal lines. A short descriptive title should appear above each table, with possible legend and footnotes (identified with a, b, c, etc.) below.
Figures should be line drawings in black ink. Figures should be completely labeled, the size of the lettering being appropriate to that of the drawing, taking into account the necessary reduction in size. All legends should be typed double-spaced on separate sheets. If figures are not to be reduced their format should not exceed 16.0 x 20.2 cm. Colour reproduction is possible. Authors wishing to publish colour figures will be expected to pay for their production costs. Figure legends have to be submitted on separate sheets.

Equations have to be numbered consecutively.

Nomenclature. A list of symbols and abbreviations (e.g., of enzymes) should be provided. 'Fermentation' and 'fermenter' have become very ambiguous expressions and, therefore, should be avoided in this Journal. Preferably use other expressions such as cultivation or

bioreactor, respectively. Units and Dimensions should be expressed according to IUPAC nomenclature, e.g.

Time, s, min, h, d, a; Mass, ng, mg, mg, g, kg, t; Length, nm, mm, mm, cm, m, km; Volume, l, ml, ml; Dalton, Da, for molecular mass. Molecular weight has no dimension. Negative powers should be used instead of fractions, e.g., g l-1 h-1, nmol ml-1, etc. Dimensions should not be mixed with specifications, e.g., protein per biomass (g g-1) instead of g protein/g biomass.

Scientific and Engineering Symbols. Growth kinetics and cultivation: As recommended by the International Commission at the 2nd Int. Symposium on Cont. Cultivation of Microorganisms, Prague 1962 (Proceedings published by Academic Press, New York, p. 379, 1962). Other symbols: as per 'Perry's Chemical Engineering Handbook'.