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UTILIZAÇÃO DE RESÍDUOS DO BENEFICIAMENTO DE CAMARÕES CULTIVADOS PARA OBTENÇÃO DE NOVOS INSUMOS PARA AQUICULTURA

Recife
2016

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Data da Aprovação 29/02/2016

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Tabacaria (resíduo)
- Fernando Pessoa -

...
Estou hoje vencido, como se soubesse a verdade.
Estou hoje lúcido, como se estivesse para morrer,
E não tivesse mais irmandade com as coisas
Senão uma despedida, tornando-se esta casa e este lado da rua
A fileira de carruagens de um comboio, e uma partida apitada
De dentro da minha cabeça,
E uma sacudidela dos meus nervos e um ranger de ossos na ida.

Estou hoje perplexo, como quem pensou e achou e esqueceu.
Estou hoje dividido entre a lealdade que devo
À Tabacaria do outro lado da rua, como coisa real por fora,
E à sensação de que tudo é sonho, como coisa real por dentro.

Falhei em tudo.
Como não fiz propósito nenhum, talvez tudo fosse nada.
A aprendizagem que me deram,
Desci dela pela janela das traseiras da casa.
Fui até ao campo com grandes propósitos.
Mas lá encontrei só ervas e árvores,
E quando havia gente era igual à outra.
Saio da janela, sento-me numa cadeira. Em que hei de pensar?

Que sei eu do que serei, eu que não sei o que sou?
Ser o que penso? Mas penso tanta coisa!
E há tantos que pensam ser a mesma coisa que não pode haver tantos!
Gênio? Neste momento
Cem mil cérebros se concebem em sonho gênios como eu,
E a história não marcará, quem sabe?, nem um,
Nem haverá senão estrume de tantas conquistas futuras.
Não, não creio em mim.
Em todos os manicômios há doidos malucos com tantas certezas!
Eu, que não tenho nenhuma certeza, sou mais certo ou menos certo?
...

À minha mãe Loide Lopes, avó Ester Laurentino e irmã

Rafaella Lopes. Minhas forças.

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RESUMO

A aquicultura é um dos mais promissores agronegócios em desenvolvimento no mundo. A utilização de resíduos do processamento de organismos aquáticos é considerada uma atividade necessária, pois além de diminuir a quantidade de resíduos biológicos depositados em aterros e cursos d'água também representa uma importante fonte de moléculas bioativas de valor agregado, permitindo que a aquicultura seja uma atividade sustentável ecologicamente e economicamente. No processamento de camarões, aproximadamente 30-70% do peso total do animal é considerado resíduo, embora ainda possua uma considerável quantidade de proteínas de músculo, pigmentos, lipídeos e alguns carboidratos e polissacarídeos como a quitina. Esses materiais podem ser recuperados e aplicados como novos produtos de valor agregado ou simplesmente abastecendo a estrutura de cultivo local. Neste trabalho, utilizou-se o resíduo do beneficiamento dos camarões *Litopenaeus vannamei* e *Macrobrachium rosenbergii*, para a obtenção de insumos para a aquicultura. Espécimes foram obtidos de carcinocultores locais e descabeçados manualmente, o material foi acondicionado em sacos plásticos contendo 1 kg e acondicionados a -20°C até serem utilizados. Triturou-se com água destilada (1:1) e este homogenato foi analisado quanto a capacidade proteolítica e então submetido à autólise enzimática por 30 min. e 2h a 45°C sob agitação. Após esse tempo, o material foi aquecido a 100°C por 10 min. para inativação enzimática e então filtrado para separação de fases. A fase líquida do processo realizado com a espécie *L. vannamei* foi misturada ao farelo de soja para originar quatro diferentes bioprodutos. Desses bioprodutos foram realizadas análises microbiológicas específicas e de mesófilos, obtenção de composição chemical, aminograma, composição de ácidos graxos e testes de inibição proteolítica. Do líquido do *M. rosenbergii* foram obtidos os níveis proteícos, de lipídeos, cinzas e carboidratos. A parte sólida (carapaça) foi separada e extraída com etanol 90% para delipidação e obtenção de carotenoides. O resíduo sólido foi tratado para obtenção de quitina e produção de quitosana.

Palavras-chave: *Litopenaeus vannamei. Macrobrachium rosenbergii*. Resíduos. Fonte proteica.

ABSTRACT

Aquaculture is one of the most promising agribusiness development in the world. The use of the processing of aquatic waste is considered a necessary activity because in addition to reducing the amount of organic waste going to landfill and water streams also represents an important source of bioactive molecules of added value, enabling aquaculture is one ecologically and economically sustainable activity. In the processing of shrimp approximately 30-70% of the total weight of the animal is considered as waste while still have a considerable amount of muscle proteins, pigments, lipids and some carbohydrates and polysaccharides such as chitin. These materials can be recovered and applied as new value-added products or just supplying the local farming structure. In this work, we used the processing of the waste from shrimp *Litopenaeus vannamei* and *Macrobrachium rosenbergii*, to obtain supplies for aquaculture. Specimens were obtained from local carcinocultores and headless manually, the material was placed in plastic bags containing 1 kg, and stored at -20°C until used. Trituration with distilled water (1: 1) and this homogenate was assayed for proteolytic capacity and then subjected to enzymatic autolysis for 30 min. and 2h at 45 ° C under stirring. After this time the material was heated at 100 ° C for 10 min. for enzyme inactivation and then filtered to separate the phases. The liquid phase of the process carried out with the L. vannamei species was mixed with soybean meal to yield four different bioproducts. These products were made specific microbiological testing and mesophilic, obtaining chemical composition, aminogram, fatty acid composition and proteolytic inhibition tests. The liquid *M. rosenbergii* were obtained-protein levels, lipid, ash and carbohydrates. The solid portion (the carapace) was separated and extracted with 90% ethanol to delipidation and obtain carotenoids. The solid residue was treated to obtain chitin and chitosan production.

Keywords: *Litopenaeus vannamei. Macrobrachium rosenbergii*. Processing waste. Protein source.

LISTA DE ABREVIAÇÕES

ºC - Graus Celsius

µL - Microlitros

ANOVA - Anállise de variância

AOAC - Official methods of analysis

BHAP- Bactéria heterotrófica aeróbica psicrotrófica

BP - Bioproduto

ANVISA - Agência Nacional de Vigilância Sanitária

CFU - Colony forming units

DM - Dry Matter

EAA - Essential amiono acids

FAO - Food and Agriculture Organization

FT-IR Espectroscopia de Infra Vermelho por Transformada de Fourier

g - Gramas

HCl - Ácido Clorídrico

HUFA - Highly unsaturated fatty acids

IAAI - Indispensable Amino Acid Index

ICMSF - International Commission on Microbiological Specifications for Foods

kg - Quilograma

LEAAL - Laboratório de Experimentação e Análise de Alimentos

M - Molar

mL - Mililitros

mm - Milímetro

mM - Milimolar

MPA - Ministério da Pesca e Aquicultura

MPN - Most Probable Number

NaOH - Hidróxido de Sódio

ND - Not detected

nm - Nanometros

NRC - Nutrient Requirements of Fish and Shrimp

PAC - Aluminum polychloride

PCA - Plate Count Agar

pH - Potencial Hidrogeniônico

rpm - Rotações por minutoTCA - Trichloroacetic Acid

TP - Test Protein

Tris - 2-Amino-2-(hidroximetil)-1,3-propanodiol

WEP - Whole-egg protein

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1. Introdução

A aquicultura é a prática de cultivo de organismos que apresentam, pelo menos, uma parte do seu ciclo de vida em ambientes aquáticos. Essa prática possui as seguintes classificações: piscicultura - cultivo de peixes de águas doce e salgada; malacocultura - cultivo de moluscos; carcinicultura - criação de camarões; algicultura - produção de algas; ranicultura - criação de rãs; reptilcultura - criação de jacarés (MPA, 2013).

A alimentação consiste num dos fatores mais importantes do cultivo de peixes. Através do alimento, os animais obtêm a energia necessária para sintetizar moléculas requeridas para o desenvolvimento, sobrevivência e realizar ações tais como: locomoção, reprodução e defesa. A farinha de peixe é a principal fonte proteica dietária que satisfaz as exigências dos aminoácidos essenciais e não essenciais na produção de ração para a aquicultura (TACON, 2007).

A maioria das farinhas comerciais de peixe é produzida a partir de várias espécies e pode ser rotulada em função da cor (branca ou marrom), espécie de pescado, procedimento de manufatura ou país de origem. A qualidade destas farinhas depende de vários fatores, tais como, método de captura do pescado, temperatura e tempo de estocagem antes do processamento, e composição dos componentes do pescado processados (OLIVEIRA, 2002). Apesar de ser um ingrediente de alto valor protéico, a sua grande participação na composição dos custos das rações tem conduzido ao interesse contínuo na identificação e desenvolvimento de novas fontes alternativas de proteínas.

O crescimento da indústria de pescado tem gerado uma grande quantidade de resíduos e subprodutos que representam um desafio para os empresários do setor e para a comunidade científica especializada, que buscam estratégias para que essa atividade seja sustentável e ecologicamente viável (BEZERRA et al., 2001). A produção de farinha ou hidrolisado protéico a partir de subprodutos das indústrias pesqueiras representa uma excelente alternativa para o incremento da oferta de proteína animal (MACKIE, 1982; HAARD, 1992; KENT, 1997), já que estes subprodutos são usualmente descartados. Devido ao grande volume de camarões cultivado e capturado, a

farinha de resíduos tem sido identificada como uma fonte de proteína animal de grande potencial (FANIMO et al., 2000), podendo contribuir ainda para a redução de problemas ambientais pela inadequada destinação destes resíduos no processamento (HEU et al., 2003). Uma das possíveis soluções é transformar esses resíduos em material para uso na formulação de rações para animais, inclusive peixes (CAVALHEIRO et al., 2007). No processamento do camarão, geralmente são removidas a cabeça, o exoesqueleto e a porção posterior. Estes subprodutos correspondem a, aproximadamente 52% do seu peso total, o que torna importante seu aproveitamento, do ponto de vista econômico, industrial e ambiental (HEU et al., 2003).

Silva (2006) elaborou um hidrolisado protéico a partir de cabeças de camarão marinho *Litopenaeus vannamei*, por autólise enzimática, obtendo-se um concentrado protéico, o qual foi considerado uma excelente fonte alimentar, sobretudo de aminoácidos.

Existem várias metodologias para a obtenção de hidrolisado protéico a partir de produtos e subprodutos pesqueiros (GILDBERG, 1993). A presença de enzimas proteolíticas no trato digestório de animais aquáticos tem uma influência significante na produção de hidrolisados. Essa produção pode ser realizada através do processo de autólise ou por um método de hidrólise, através da adição de enzimas (SHAHIDI et al., 1995). Gildberg e Stenberg (2001) demonstraram que a proteína dos subprodutos do camarão pode ser hidrolisada por proteases comerciais e recuperada como hidrolisado protéico com alto conteúdo de aminoácidos essenciais.

Com isso, o objetivo do trabalho foi a obtenção de alguns insumos, de potencial uso na aquicultura, a partir de rejeitos da indústria de beneficiamento de camarão.

2. Objetivos

2.1. Objetivo Geral

Obter insumos passíveis à utilização pela aquicultura a partir de resíduos do beneficiamento de camarões cultivados.

2.2. Objetivos Específicos

- Obter hidrolisados proteicos dos camarões *Litopenaeus vannamei* e *Macrobrachium rosenbergii;*
- Produzir novos bioprodutos a partir da mistura do hidrolisado do camarão *L. vannamei* e farelo de soja;
- Determinar a composição centesimal, aminograma e composição de ácidos graxos dos bioprodutos;
- Avaliar a inibição proteolítica de algumas espécies de peixes importantes para aquicultura na presença dos bioprodutos e seus componentes;
- Extrair moléculas bioativas da carapaça do *M. rosenbergii* e produzir quitosana;
- Avaliar a capacidade floculante da quitosana obtida do *M. rosenbergii* sob espécies de microalgas cultivadas.

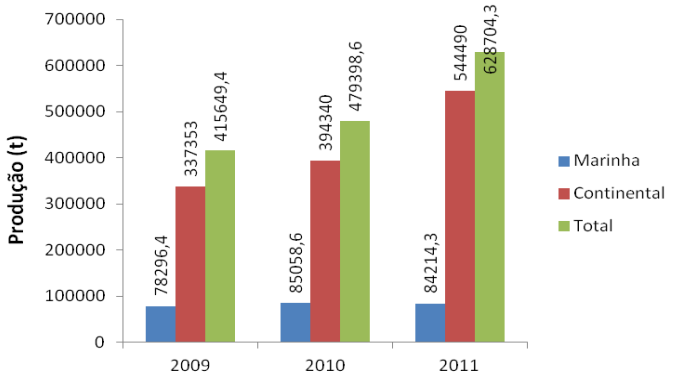
3. Revisão Bibliográfica

3.1. Aquicultura e o Panorama da produção de pescados

A aquicultura é um setor que tem crescido em média 9% ao ano, principalmente devido a alta demanda mercadológica que se mostra superior à capacidade de captura do pescado (GATLIN et al., 2007). De acordo com Hardy (2009), a captura não produz anualmente a quantidade necessária para garantir a demanda de consumo, sendo assim a aquicultura deve-se coadunar com a captura de pescado. Para espécies marinhas, entre 1974 e 2011 a captura sustentável caiu de 90% para 71%, sendo, atualmente, considerada não sustentável, o que provoca a sobrepesca (FAO, 2014).

Em 2012, a aquicultura mundial produziu mais de 66 milhões de toneladas de pescado. Nesse cenário uma das maiores representantes de produção é a China, com mais de 43 milhões de toneladas de pescado. Segundo o Ministério da Pesca e Aquicultura (2013), a aquicultura brasileira, em 2011, produziu mais de 600 mil toneladas de pescado, representando um aumento de 51,2% na produção entre os anos de 2009-2011 (Figura 1).

Figura 1: Produção de pescado no Brasil, no ano de 2011. Adaptada MPA, 2013.



Mundialmente, no ano de 2012, a captura das principais espécies de camarão chegou ao seu máximo de 3,4 milhões de toneladas (FAO, 2014). No Brasil, a produção de camarões, oriundos da captura e da aquicultura, chegou a somar mais de 108 mil

toneladas (MPA, 2013). Estima-se que 52% do total dessa produção brasileira, mais de 56 mil toneladas, originem os subprodutos do beneficiamento de camarão - cabeça, carapaça e cauda (HEU et al., 2003).

3.2. Ingredientes Proteicos

As rações produzidas comercialmente possuem uma gama de variedades de formas e composição específicas às necessidades de diferentes espécies de peixes e suas fases de cultivo. As dietas específicas para peixes carnívoros, como o pintado (*Pseudoplatystoma corruscans*), o dourado (*Salminus brasiliensis*) e a truta arco-íris (*Oncorhynchus mykiss*) possuem um maior teor de proteína, entre 40 e 48%, comparadas às rações para peixes onívoros como as tilápias (*Oreochromis niloticus*), carpa comum (*Cyprinus carpio*), pacu (*Piaractus mesopotamicus*), tambaqui (*Colossoma macropomum*), entre outros, que variam entre 24 e 32% de proteína. Na avaliação da qualidade das dietas utilizadas para alimentação de peixes, o teor de proteína é um dos parâmetros mais utilizados (KUBITZA; CYRINO; ONO, 1998).

Nas décadas de 1980 e 1990, ocorreram mudanças climáticas que diminuíram drasticamente a pesca das espécies de peixes responsáveis pela fabricação da farinha de peixe. Quando a pesca dessas espécies caiu, o preço desse ingrediente farinha de peixe subiu, acarretando numa diminuição da porcentagem desse ingrediente na formulação das rações e um aumento na utilização de fontes proteicas alternativas. Em 2006, além dos fenômenos naturais, uma baixa na disponibilidade natural das espécies, utilizadas no fabrico da farinha de peixe e um aumento na demanda desse ingrediente pela aquicultura, fez com que o preço dessa farinha aumentasse ainda mais. Esse fato, mostrou que a produção anual de farinha de peixe não é suficiente para a demanda da aquicultura, havendo uma diminuição na utilização desse ingrediente (HARDY, 2009).

Dentre as fontes de proteína de origem vegetal, a soja *Glycine max* (L) é considerada, a nível global, como a opção com maior potencial para substituir a farinha de peixe na formulação das rações comerciais, pois apresenta um alto teor de proteínas, baixos teores de carboidratos e fibras, alta digestibilidade, e bom padrão de aminoácidos essenciais quando comparados a outras fontes de proteína vegetal (ALAM et al., 2005).

3.3. Nutrientes necessários na alimentação de peixes

A formulação de rações de alto valor nutricional e boa digestibilidade depende do conhecimento fisiológico da digestão da espécie de peixe a ser cultivada, como também de seu manejo alimentar e da exigência dos nutrientes essenciais, em especial as exigências energéticas, de proteínas e aminoácidos (PORTZ; FURUYA, 2013). Essa alimentação utilizada em cultivos intensivos de peixes chega a representar 50% do total dos custos, destes a proteína é o nutriente que representa maior valor econômico (PEREIRA et al., 2012). Esses nutrientes irão afetar o crescimento, a eficiência dietética, a composição corporal, a resposta econômica e os impactos ambientais do manejo desses animais (PORTZ; FURUYA, 2013).

A energia é necessária a todos os processos fisiológicos dos peixes e eles a adquirem na alimentação na forma de gordura, proteína e carboidrato (KAUSHIK e MÉDALE, 1994). Essa energia necessária ao metabolismo dos peixes é basicamente oriunda das fontes de lipídeos, além das fontes proteicas, já os carboidratos estão presentes em menores quantidades nas dietas ofertadas por possuírem menor representação energética (TOCHER, 2003).

3.3.1. Proteínas e aminoácidos

As proteínas são os principais constituintes orgânicos dos peixes, chegando a representar 75% do total da matéria seca corporal. Essas proteínas possuem várias funções: estrutural (músculos, queratina e colágeno), metabólica (na forma de enzimas e hormônios), transportadora de substâncias e participante do sistema de defesa do organismo. Esse nutriente, quando digerido, libera aminoácidos livres que serão absorvidos e formarão novas proteínas ocasionando o crescimento e reparando tecidos e órgãos danificados (PORTZ; FURUYA, 2013).

A concentração ideal de proteínas em rações para peixes depende do balanço entre a energia digestível e a proteína bruta (PORTZ; FURUYA, 2013). Uma dieta deficiente energeticamente resulta em uma baixa taxa de crescimento, pois a proteína ingerida será utilizada para produção de energia, já uma alta concentração energética na alimentação irá originar peixes gordurosos, característica não desejada em peixes

cultivados (NRC, 1993, 2011). Porém, quando a alimentação tem um nível proteico mais elevado que o necessário, haverá um gasto energético extra para que os aminoácidos ingeridos em maior quantidade sejam metabolizados (PORTZ; FURUYA, 2013).

Por muitos anos as formulações de rações se baseavam nas necessidades de proteína bruta do animal, essas necessidades são bastante variáveis entre as espécies e idade do animal (Tabela 1). Porém, a evolução no conhecimento fez com que a inserção de proteína não esteja apenas baseada na proteína bruta, mas sim no balanceamento da exigência dos aminoácidos essenciais de cada espécie, se suprindo as necessidades desses aminoácidos, e se adicionando industrialmente aqueles que se apresentam deficientes (PORTZ; FURUYA, 2013).

3.3.2. Lipídeos

São moléculas quimicamente diversas que possuem como característica em comum a insolubilidade em água (NELSON E COX, 2011). Os lipídeos são importantes por possuírem diversas funções, dentre elas pode-se citar: são uma das principais fontes de energia para os peixes; mantêm a estrutura, estabilidade e permeabilidade das membranas plasmáticas; são a fonte de ácidos graxos essenciais; são precursores dos hormônios (GARCIA et al., 2013).

A literatura sobre lipídeos para peixes é incompleta, muitas informações sobre o metabolismo dessas macromoléculas em nutrição de peixes é desconhecida ou deduzida do conhecimento produzido para mamíferos. Além de que, grande parte das pesquisas atuais sobre a nutrição lipídica em peixes foi realizada em peixes de águas frias ou temperadas, portanto sendo ainda menores os resultados para peixes de águas tropicais. Quando fala-se das exigências nutricionais dessas moléculas em dietas, para peixes e mesmo outros animais, são pouquíssimo conhecidas (GARCIA et al., 2013).

Tabela 1: Requerimento proteico para algumas espécies de peixes de interesse econômico.

Espécie/ Nome comum	Peso corporal (g)	Requerimento de Proteína Bruta (%)	Hábito Alimentar	Referência
Oreochromis niloticus/ Tilápia do Nilo	0,8-15,5	28	Onívoro	Bomfim et al., 2008
Oreochromis niloticus/ Tilápia do Nilo	100 - 500	25,11	Onívoro	Righetti et al., 2011
Arapaima gigas/ Pirarucu	120	48,6	Carnívoro	Ituassú et al., 2005
Cichla sp./ Tucunaré	10-30	37-41	Carnívoro	Sampaio et al., 2000
Pseudoplatystoma fasciatum/Cachara	53-54	39	Carnívoro	Castro Silva, 2013
Sciaenops ocellatus/ Corvinão de pintas	2	40	Carnívoro	Serrano et al., 1992
Ictalurus punctatus/ Bagre do canal	27	24	Onívoro	Robinson e Li, 1997
Rhamdia quelen/ Jundiá	131,4	50	Onívoro	Camargo et al., 2005
Leporinus macrocephalus/ Piavuçu	0,625	34	Onívoro	Feiden et al., 2009

Proteínas, carboidratos e lipídeos são utilizados na obtenção de energia, porém os lipídeos possuem um maior valor calórico quando comparado com essas outras biomoléculas, sendo assim, são mais eficientes na obtenção energética (GLENCROSS, 2009; BUREAU et al., 2002). Em espécies de peixes carnívoras marinhas os lipídeos são a principal fonte energética pois esses animais possuem uma baixa capacidade de utilizar carboidratos para obter energia (SMITH, 1989).

O nível ideal de lipídeos em dietas variam a partir de diversos fatores: o habitat natural da espécie a ser cultivada, o hábito alimentar dela, a temperatura das águas, a fase de desenvolvimento do animal, quantidade de proteína da ração e até a quantidade de carboidrato presente na dieta. De um modo geral, considera-se que inserções de 10 a 20% de lipídeos nas rações farão com que a proteína seja bem utilizada para crescimento e não haverá um grande acúmulo de gordura nos tecidos (GARCIA et al., 2013).

Todos os seres vivos sintetizam ácidos graxos, como os ácido palmítico (16:0) e o ácido esteárico (18:0) (NELSON; COX, 2011). Porém, nenhum vertebrado consegue sintetizar o ácido linoléico (18:2 n-6) e o linolênico (18:3 n-3), sendo estes, portanto,

considerados ácidos graxos essenciais (NRC, 2011). Uma dieta deficiente em ácidos graxos essenciais pode estacionar o crescimento dos animais e aumentar a mortalidade no cultivo, pode, também, ocasionar patologias como erosão das nadadeiras, palidez, aumento no volume do fígado, redução no potencial reprodutivo, entre outras (NRC, 1993; SARGENT, et al., 2002; GLENCROSS, 2009).

3.3.3. Carboidratos e Fibras

Para carboidratos, não existe uma exigência de inclusão em dietas para peixes, porém a ausência desse nutriente ocasiona o catabolismo de proteínas e lipídeos para a síntese de energia. Com isso, incluir adequadamente carboidratos na ração evita que a proteína seja utilizada para gerar energia, além de minimizar a liberação de compostos nitrogenados na água (FRACALOSSI; RODRIGUES; GOMINHO-ROSA, 2013).

Os carboidratos presentes em ingredientes vegetais possuem, a grosso modo, amido e fibras. Após a ingestão do amido haverá uma quantidade maior de glicose disponível para o peixe por algumas horas, essa glicose então poderá ser usada como fonte imediata de energia, pode ser estocada na forma de glicogênio, originar outros compostos (como aminoácidos não essenciais) ou ser excretada (FRACALOSSI; RODRIGUES; GOMINHO-ROSA, 2013).

Desde os anos 50 diversos autores elaboraram definições para fibra alimentar. Em 1953, Hipsley definiu fibra como constituinte das paredes celulares que não são digeríveis. Para a FAO (1998) a fibra dietária é um material comestível, de origem vegetal ou animal, que não consegue ser hidrolisada por enzimas digestórias humanas. Segundo a Agência Nacional de Vigilância Sanitária, a fibra alimentar é qualquer material comestível que não será hidrolisado pelas enzimas endógenas do trato digestório humano (BRASIL, 2003).

3.4. Digestão em peixes

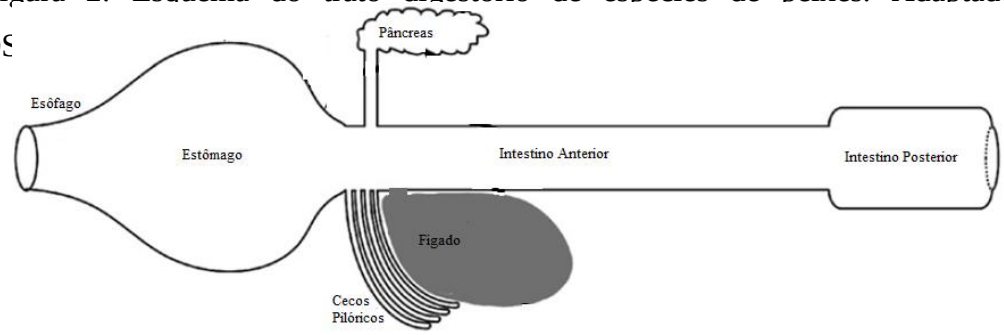
O conhecimento sobre a dieta e os hábitos alimentares é essencial na compreensão de vários aspectos da biologia dos peixes sendo muito importante para a aplicação na aqüicultura e na formulação de rações. Nas últimas décadas numerosos

trabalhos mostraram os conteúdos estomacais de diversas espécies de peixes, evidenciando as diferentes fontes de nutrientes utilizadas por eles (fito e zooplânctons, macroalgas, plantas aquáticas, insetos, crustáceos, moluscos, outros peixes, aves, mamíferos, entre outros). De acordo com seu hábito alimentar os peixes foram categorizados em: herbívoros, onívoros ou carnívoros/piscívoros (GROSSELL et al., 2011).

Segundo Pillay (1952) a natureza do alimento ingerido depende primeiro da morfologia do trato digestório e do comportamento alimentar do peixe e segundo da composição e quantidade do alimento que está disponível no meio. As estruturas macroscópicas e microscópicas dos órgãos digestórios se relacionam com a natureza dos alimentos e a forma como eles são ingeridos. Com isso, o comprimento do intestino tem relação com o hábito alimentar do animal (CHAVES E VAZZOLER, 1984).

Em aquicultura a formulação das rações é uma etapa muito importante. Várias características, como tamanho do pélete, consistência e palatabilidade, têm que ser levadas em consideração para que a ração seja atrativa para o animal. Quanto à morfologia do tubo digestório dos peixes elucidam questões da organização espacial dos órgãos e a sua relação com dados fisiológicos e bioquímicos (Fig. 2) (GROSSELL et al., 2011).

Figura 2: Esquema do trato digestório de espécies de peixes. Adaptado de GROS



Anatomicamente a divisão esôfago e estômago não é claramente demarcada. Os cecos pilóricos, órgão que não está presente em todos os peixes, possuem células muito parecidas com as do intestino e apresenta as funções de digestão e absorção. Além da digestão, o trato digestório nos peixes tem a função de osmorregulação, respiração e defesa (GROSSELL et al., 2011). As espécies de peixes diferem bastante na capacidade

de digestão. Esta variação reflete diferenças anatômicas e funcionais do trato gastrointestinal e dos órgãos associados. Os peixes onívoros possuem funções digestivas capazes de hidrolisar uma variedade maior de alimentos quando comparados com os peixes carnívoros (ALMEIDA et al., 2006).

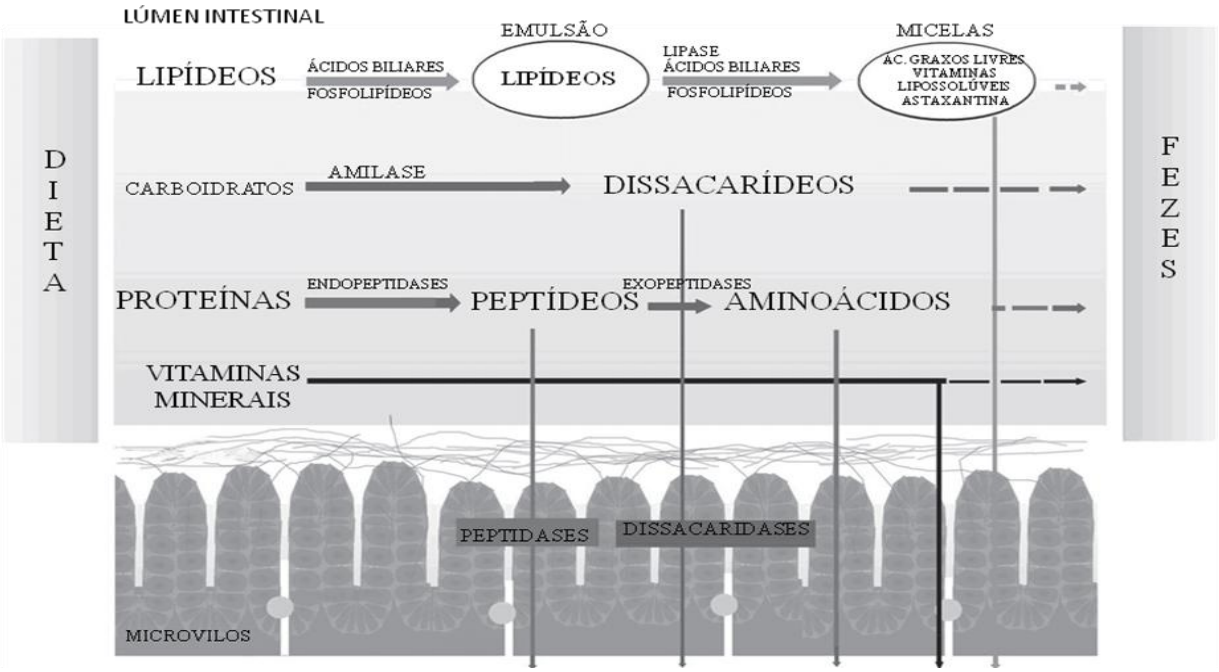
No estômago começa a quebra física e enzimática do alimento. Neste órgão a secreção do HCl converte o pepsinogênio em pepsina e inicia a hidrólise das proteínas (YUFERA et al., 2004; WU et al., 2009; GROSSELL et al., 2011). Quando o bolo alimentar segue para o intestino a ação das enzimas secretadas pelos órgãos acessórios (pâncreas e fígado) continuam o processo de hidrólise das proteínas até peptídeos e irão fazer a digestão das gorduras e carboidratos para que possam ser absorvidos pelas células intestinais (Figura 3) (GROSELL et al., 2011).

Na produção das rações os ingredientes protéicos de origem animal, como farinhas de peixe e de lula, possuem um alto percentual protéico, porém apresentam custos elevados (MENDOZA et al., 2001). Os ingredientes de origem animal apresentam um balanceamento ideal entre aminoácidos e outros nutrientes essenciais (LEMOS; EZQUERRA E GARCÍA-CARREÑO, 2000), porém para que os custos com esses ingredientes não sejam muito altos são utilizados na fabricação de rações, como matéria prima, subprodutos oriundos do processamento de pescados, como farinhas de ossos, vísceras e escamas (HUGHES, 2001).

As proteínas de origem vegetal, como o farelo de soja, são largamente utilizadas na formulação de rações, pois diminuem os custos com a produção da ração, porém elas apresentam menores índices protéicos e uma variedade de fatores anti-nutricionais (PIKE E HARDY, 1997).

Uma vez que todos os ingredientes utilizados na formulação das rações irão influenciar diretamente na digestão do animal, é necessário que a matéria prima utilizada nas rações não seja de tão baixa qualidade para que os animais possam digerir e absorver os nutrientes.

Figura 3: Esquema do processo de digestão no trato digestório dos peixes (GROSELL et al., 2011).

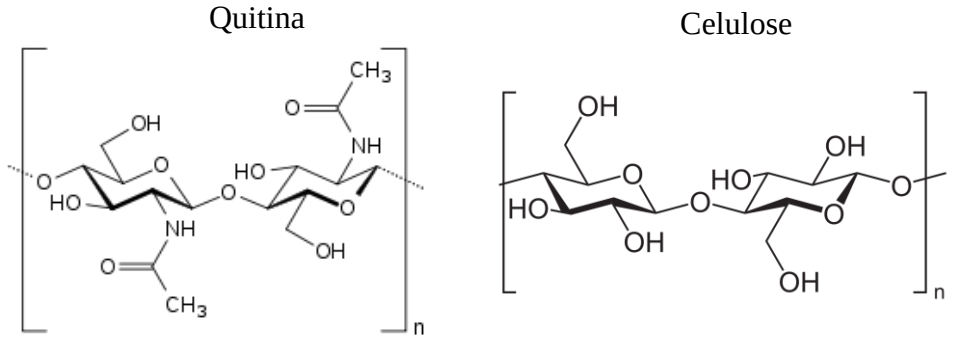


3.5. Quitina e Quitosana

Uma das formas de agregar valor aos resíduos de camarão é a extração de quitina de sua carapaça para posterior produção de quitosana. A quitina e a quitosana possuem várias utilizações, dentre elas pode-se citar: agente antioxidante, cicatrização óssea, agente redutor de colesterol, produção de filmes comestíveis, agente floculante, estimulante de crescimento vegetal, estimulante do sistema imune, imobilização de células, produção de colunas cromatográficas, entre outras (DATTA, BASU E DATTA, 1984; KRAJEWSKA, 2004; RINAUDO, 2006; KIM, 2013).

A quitina é o segundo biopolímero mais abundante na natureza, sendo a celulose o mais abundante. Essas duas moléculas se diferem pela presença de um grupo acetoamino na quitina em substituição a uma hidroxila da celulose (Fig. 4) (DUTTA et al., 2015). A carapaça de camarões pode ter em sua composição até 11% de quitina. Além dos crustáceos, essa molécula também é encontrada no exoesqueleto dos insetos e na parede celular dos fungos (SYNOWIECKI E AL-KHATEEB, 2000).

Figura 4: Estrutura da quitina e da celulose.



A quitosana é um polissacarídeo composto por ligação β(1→4) de N-acetil-D-glicosamina e D-glicosamina obtido da hidrólise alcalina do grupamento N-acetil da quitina (Fig. 5), esse processo pode ser realizado comercialmente numa faixa entre 80 e 85% de desacetilação (Fig. 6) (CAHÚ, 2014).

A quitina tem os grupamentos acetamido nos resíduos de N-acetil-glucosamina e a quitosana tem como predominância os grupamentos amino na glucosamina. Quando o grau de desacetilação (GD) é inferior a 0,5 o polímero é considerado quitina e acima de 0,5 tem-se a quitosana (Fig. 7). A quitina pode ser encontrada na natureza parcialmente desacetilada, esse composto têm microestrutura predominantemente cristalina, o que não permite dissolução na maioria dos solventes, representando um dos maiores problemas relacionado ao seu uso. Porém a quitosana apresenta estrutura menos cristalina que a quitina, pois perdeu grupos acetis, o que ocasiona uma maior solubilidade em meio aquoso, embora não seja solúvel em valores de pH neutros e alcalinos (RINAUDO, 2006).

Figura 5: Estrutura da quitina e quitosana.

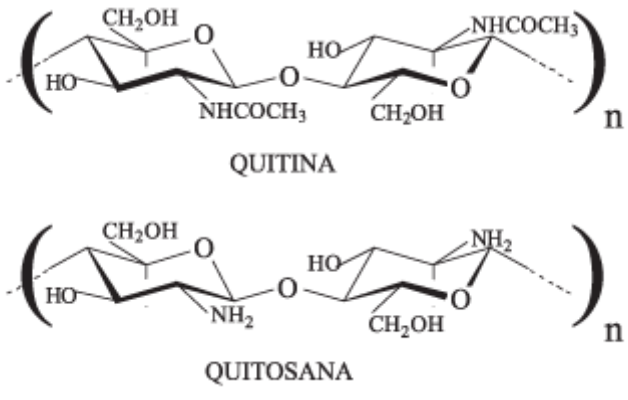


Figura 6: Processo de desacetilação da quitina em quitosana. Adaptado Kumar, 2000.

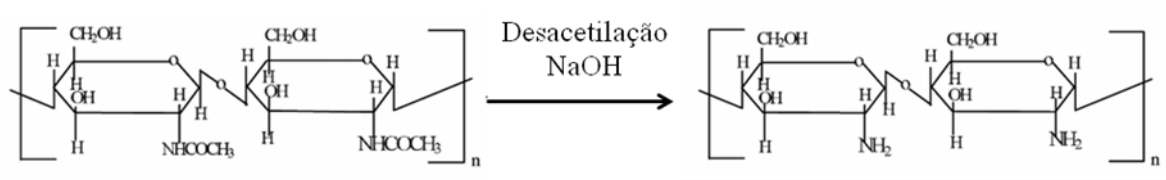
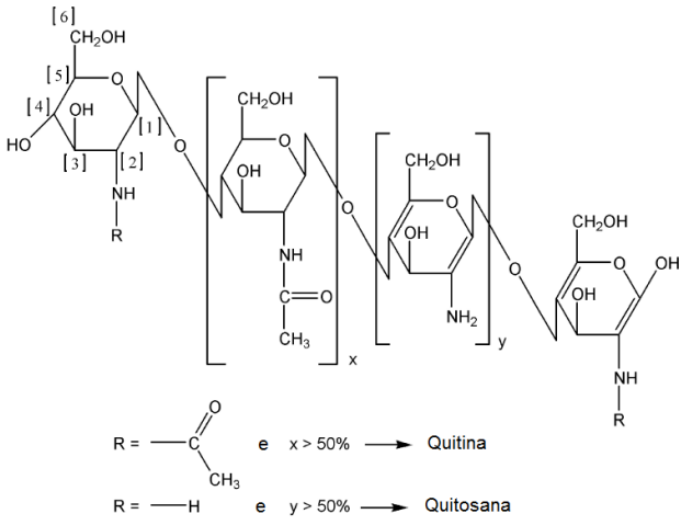
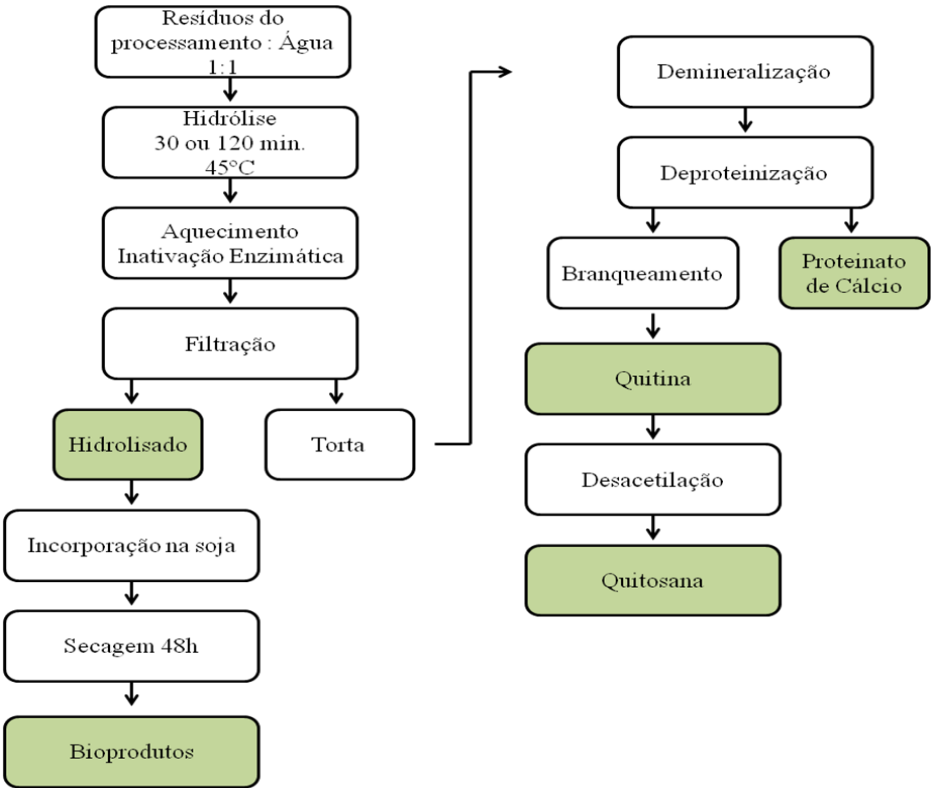


Figura 7: Estrutura do polímero de N-acetil glucosamina e glucosamina, dependendo do grau de desacetilação é denominado quitina (GD%<0,5) ou quitosana (GD%>0,5).



A partir dos resíduos oriundos do processamento dos camarões foi elaborado um processo de recuperação de biomoléculas e produção de bioprodutos destinados à aquicultura (CAHÚ, 2014). A figura 8 apresenta um esquema que resume as etapas do processo. Após todas as etapas obtem-se hidrolisados proteicos, bioprodutos, quitina, quitosana e um proteinato de cálcio.

Figura 8: Esquema do processo de recuperação de biomoléculas e produção de bioprodutos a partir de resíduos do processamento de camarões cultivados. Adaptado de Cahú et al., 2012.



3.6. Os camarões *Litopenaeus vannamei* e *Macrobrachium rosenbergii*

A classificação taxonômica do *L. vannamei*, segundo Pérez-Farfante (1997), e do *M. rosenbergii*, por De Grave et al. (2013), está apresentada na Tabela 2.

Por ser a principal espécie de camarão cultivada no mundo, o *L. vannamei* (Figura 9A) é uma espécie de camarão muito bem estudada (TACON, 2007). Caracteriza-se pelo rostro serrilhado com um ou dois dentes e oito a nove dentes dorsais (HENDRICKX, 1996). Este camarão marinho é oriundo do oceano Pacífico, distribui-se de Sonora, no México, até o norte do Peru, em águas com profundidade máxima de 72 m (HOLTHUIS, 1980). Além do Pacífico essa espécie de camarão pode ser encontrada em vários países, onde foi introduzida em sistemas de cultivo semi-intensivo e intensivo. Umas das vantagens dessa espécie é a sua adaptabilidade a águas turvas e

com baixa concentração de oxigênio. Durante seu desenvolvimento, este camarão tende a apresentar um hábito alimentar onívoro, demonstrando alta capacidade de digerir proteínas de fontes vegetais (CUZON et al., 2004).

Tabela 2: Classificação taxonômica das espécies de camarão *Litopenaeus vannamei* (PÉREZ-FARFANTE, 1997) e *Macrobrachium rosenbergii* (DE GRAVE et al., 2013).

Espécie / **Táxon**	***Litopenaeus vannamei***	***Macrobrachium rosenbergii***
Reino	Animalia	Animalia
Filo	Arthropoda	Arthropoda
Subfilo	Crustacea	Crustacea
Classe	Malacostraca	Malacostraca
Ordem	Decapoda	Decapoda
Superfamília	Penaeoidea	Palaemonoidea
Família	Penaeidae	Palaemonidae
Gênero	*Litopenaeus*	*Macrobrachium*

A desova de *L. vannamei* acontece em mar aberto, no período noturno (BARBIERI JÚNIOR E OSTRENSKY NETO, 2002). Logo após a eclosão dos ovos, as larvas - seis fases como nauplios, três como protozoea e três como mysis (KITANI, 1986) - passam a fazer parte do plâncton e deixando-se levar pelas correntes irão habitar águas com salinidade entre 30% e 40% (CLARK, 1992). Após passar por todas as fases larvais se tornam pós-larvas. Estas são bentônicas e migram para regiões perto da costa, com menor profundidade, que possuam aporte fluvial e salinidade mais moderada. Após 2 ou 3 meses os juvenis retornam à bacia oceânica. Toda essa migração mostra a capacidade de adaptação das pós-larvas e juvenis de *L. vannamei* a grandes variações de salinidade, temperatura e pH (TRUJILLO, 1997).

O *Macrobrachium rosenbergii* (Figura 8B) foi uma das primeiras espécies de camarões a ser estudada cientificamente, tendo ilustrações datadas de 1705. É um camarão de água doce e salobra que vive em águas tropicais e subtropicais, se distribui naturalmente no sul e sudeste da Ásia e na Austrália, foi introduzido, notavelmente, no Brasil, na Tailândia e na Índia (NEW, 2002). E no Brasil é a espécie de água doce mais cultivada em escala comercial (FAO, 2010).

Esse camarão possui um rostro longo e desenvolvido com dentes na margem posterior e inferior, sua carapaça e seu abdome se mostram lisos e com o segundo par de patas longas, robustas e providas de dedos em quela (PIMENTEL, 2003). A postura dos ovos ocorre entre 6 e 20 horas depois do acasalamento, a fêmea carrega sua ninhada durante todo o período de incubação, que dura cerca de 19 dias e deve ocorrer na faixa de temperatura entre 26 e 28°C (LING, 1978). Após a eclosão as larvas migram para águas salobras e lá passam por oito estágios. Após os estágios larvais os animais se tornam pós-larva e agora começam a migração para a água doce, onde irão se tornarão adultos férteis (NEW, 2002; LING, 1978).

Figura 9: Exemplares das espécies *Litopenaeus vannamei* (A) e *Macrobrachium rosenbergii* (B).

A B

3.7. Microalgas

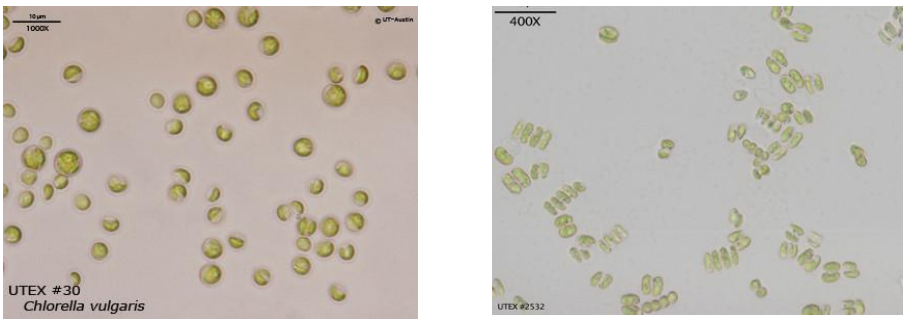
Microalgas são seres unicelulares, fotossintéticos, que habitam águas doces e salgadas e possuem grande importância econômica (LOURENÇO, 2006; MATA, MARTINS E CAETANO, 2010). Até o ano de 2009 estimou-se que existam 30000 espécies de microalgas identificadas, embora esses microorganismos sejam conhecidos há muitos séculos sua produção em larga escala está datada dos anos 1960 em países asiáticos e europeus (SPOLAORE et al. 2006; GOUVEIA et al. 2008).

Alguns gêneros, como *Chlorella*, *Dunaliella* e *Scenedesmus*, são ricos em proteínas, vitaminas e lipídeos sendo usados para alimentação de peixes, crustáceos, moluscos e da espécie humana (BRENNAN E OWENDE, 2010; MATA, MARTINS E

CAETANO, 2010). Além da utilização de microalgas na nutrição animal outras funções são bem descritas atualmente: produção de biocombustíveis (DEMIRBAS, 2010; SUALI, SARBATLY, 2012); bioindicador (PULZ E GROSS, 2004); fonte de pigmentos (LARKUM E KÜHL, 2005; Holdt & Kraan, 2011; Pangestutia & Kim, 2011); importância farmacêutica (CORNISH E GARBARY, 2010; SHIRATORI et al. 2005), entre outras.

Duas espécies de importância biotecnológica foram utilizadas nos experimentos. A espécie *Chlorella vulgaris* (Figura 10A) e a *Scenedesmus subspicatus* (Figura 10B) com classificações taxonômicas apresentadas na tabela 3.

Figura 10: Microalgas *Chlorella vulgaris* (A) e *Scenedesmus subspicatus* (B) (UTEX, 2016).



O gênero *Chlorella* geralmente se apresenta com células globulares de diâmetro entre 3 e 8 µm, algumas de suas espécies possuem tolerância a altas temperaturas podendo apresentar crescimento entre 15°C e 40°C. Em cultivos, *Chlorella vulgaris* (Figura 10A) se mostra com altas produtividades, o que a torna um dos organismos mais utilizado na produção de biomassa de algas (DANTAS, 2013). Essa espécie é muitas vezes utilizada como suplemento alimentar e também nas indústrias de cosmético e farmacêutica por apresentarem em sua composição proteínas, carotenóides, alguns imunoestimuladores, polissacarídeos diversos, lipídeos, vitaminas e alguns minerais que suprem variadas necessidades dessas indústrias (TOKUSOGLU E ÜUNAL, 2003; LIANG, SARKANY E CUI, 2009; DANTAS, 2013).

Quanto a *Scenedesmus subspicatus* (Figura 10B) também apresenta altas taxas de crescimento e é utilizada como um organismo modelo para estudos toxicológicos (BEHRA et al, 1999, MA et al., 2003, WIND & BELARGER, 2006, DAUS et al 2010,

GÜÇLÜ E ERTAN, 2012). A biomassa dessa espécie produzida em cultivo de larga escala pode apresentar um conteúdo de 50-56% de proteína (SOEDER E HEGEWALD, 1988). O perfil de ácidos graxos de *Scenedesmus spp.* torna essas espécies potenciais micro-organismos para a produção de biodiesel (Harman-Ware et al., 2013; Ramirez, 2013; Hernández et al., 2014).

Tabela 3: Classificação taxonômica das espécies de microalgas *Chlorella vulgaris* (BEYERINCK, 1890) e *Scenedesmus subspicatus* (CHODAT, 1926).

Espécie **Táxon**	***Chlorella vulgaris***	***Scenedesmus subspicatus***
Reino	Plantae	Plantae
Filo	Chlorophyta	Chlorophyta
Subfilo	Chlorophytina	Chlorophytina
Classe	Trebouxiophyceae	Chlorophyceae
Ordem	Chlorellales	Sphaeropleales
Família	Chlorellaceae	Scenedesmaceae
Gênero	*Chlorella*	*Scenedesmus*

3.8. Floculação de Microalgas

Para que o potencial biotecnológico das microalgas seja explorado é necessário que esses organismos sejam produzidos em larga escala e um dos principais gargalos dessa produção é a recuperação desses micro-organismos de seu cultivo. Os processos mais utilizados para a recuperação da biomassa são a floculação, filtração, sedimentação e centrifugação (Tabela 4). A escolha do processo dependente de características como o tamanho da célula, a densidade dessa microalga no cultivo e o valor dos produtos que serão produzidos (BRENNAN E OWENDE, 2010).

Na floculação há a formação de flocos, aglomerados de partículas em suspensão, que se tornam pesados e terminam por sofrer sedimentação (GREGORY E O'MELIA, 1989). Ela pode ocorrer de duas formas: para algumas espécies de microalgas pode acontecer naturalmente, no processo denominado autofloculação (LAVOIE E DE LA

NOÜE, 1987) ou como resposta a alterações na concentração de nitrogênio, oxigênio dissolvido ou pH do meio (MILLEDGE E HEAVEN, 2013).

Tabela 4: Processos mais utilizados para a recuperação de biomassa de microalgas cultivadas (Adaptação MILLEDGE E HEAVEN, 2013).

Processo	**Vantagens**	**Desvantagens**	**Concentração de sólidos secos (%)**
Centrifugação	Eficiente com muitas espécies de microalgas.	Altos investimentos operacionais.	10-22
Filtração	Variada gama de membranas e filtros.	Muito limitado à espécie e mais adequado a células maiores.	2-27
Sedimentação	Baixos custos, pode ser utilizada como etapa preliminar.	Depende da espécie, mais adequado a células densas, processo demorado, baixa concentração de sólidos.	0,5-3
Floculação	Grande diversidade de floculantes, custos variados.	Deposição do floculante	3-8

A maioria das microalgas possui superfície externa carregada negativamente, essas microalgas permanecem estáveis em suspensão devido a repulsão elétrica entre as células de algas e a repulsão das células e às moléculas de água (USDE, 1984; UDUMAN, DANQUAH E HOADLEY, 2010). O processo de floculação envolve a alteração da estabilidade desse sistema e pode ser provocado por substâncias inorgânicas e orgânicas. Dentre as inorgânicas tem-se as mais comuns as que possuem íons Al^{3+} e Fe^{3+}, já os polímeros catiônicos são as substâncias orgânicas mais utilizadas nos processos de floculação de microalgas (USDE, 1984; Grima et al., 2003).

Os grupos amino carregados positivamente tornam a quitosana um bom agente floculante de microalgas e sua digestibilidade e baixa toxicidade fazem dela ideal na recuperação de microalgas destinadas à alimentação humana e de animais aquáticos (LUBIAN, 1989). Além de não apresentar toxicidade a quitosana se mostra um bom floculante para espécies de microalgas de água doce e marinhas (NIGAM,

RAMANATHAN E VENKATARAMAN, 1980; LAVOIE E DE LA NOÜE, 1983; MORALES, DE LA NOÜE E PICARD, 1985; SUKENIK, BILANOVIC E SHELEF, 1988).

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5. CAPÍTULO I

Os resultados desse trabalho que estão apresentados no artigo “A high protein content meal based on shrimp processing waste and soybean meal: Potential use in replacement of fish meal and its inhibitory effect on fish digestive enzymes” (manuscrito) serão submetidos a publicação no periódico Animal Feed Science and Technology. (ISSN: 0377-8401)

A high protein content meal based on shrimp processing waste and soybean meal: Potential use in replacement of fish meal and its inhibitory effect on fish digestive enzymes

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Abstract

The ration represents the largest cost in aquatic animals farming. The protein source from these as the fish meal is one of the most important components in aquaculture. Fishmeal has been recognized as the main source of protein for its availability of essential nutrients (proteins and lipids) and its well balanced amino acid profile. The discovery of an alternative low-cost protein source and good amino acid profile is of high importance. This study aimed to formulate three products with variations in shrimp protein hydrolysate (SPH) concentrations. The hydrolysate used was obtained from shrimp *Litopenaeus vannamei* processing wastes by autolysis. The three bioproducts demonstrated excellent levels of proteins and lipids. The analysis of enzymatic inhibition demonstrated that the three bioproducts showed low inhibition of alkaline proteases of the digestive tracts of different species of fish. The chemical score shows that the essential amino acid levels of the three bioproducts are ideal for the production of balanced diets. Bioproducts based on SPH can be advantageous for its low cost and good nutritional value. Cultivation experiments are being carried out in order to evaluate the zootechnical effects of formulated diets with SPH bioproduct in farmed fish and shrimp.

Key-words: Shrimp protein; bioproduct; *Litopenaues vannamei*; enzymatic inhibition.

5.1. Introduction

It is estimated that 50% of shrimp processing industrial production corresponds to a set of residues and subproducts consist of head, tail and carapace (Islam, et al. 2004). According to the FAO (2014), in 2012, the shrimp processing generated 1.7 million tons of these subproducts worldwide. In Brazil, in 2011, the industry produced more than 19,000 tons of waste (MPA, 2013).

Previously, fish subproducts had no great relevance in the trade, and therefore its discard was considered a problem because raised the cost of production. Two decades ago there was an optimization in the use of these subproducts due to a rethink, increasingly frequent, the sustainability practices. Such a context, stimulated an raise in awareness of economic, social and environmental aspects that are involved in these subproducts (FAO, 2012).

Is known the largest cost in a fish farming is the protein source from these to the fish meal is one of the most important components of the aquaculture. This fishmeal has been recognized as the main source of protein because of its availability of essential nutrients (proteins and lipids) and its well balanced amino acid profile (Sun, et al. 2015).

The expansion of aquaculture generates a great demand in food production used in these culture systems (Gatlin, et al. 2007). Tacon (2009) provides for the production of feed for aquaculture at 36 million tons for the year 2015. In 2012, 21 million tons of fish were used for the production of fishmeal and fish oil. This significant production is declining because only 35% of fish meal was produced from fish waste in the same year (FAO, 2014).

Thus, the unstable production and the high price of fish meal made it difficult to attend the growing demand for production of the food industry for aquaculture. All this makes research directed to the replacement of fish meal by more sustainable and less costly products become priority (Sun, et al. 2015).

Several papers describe the production of a protein hydrolysate shrimp waste using outer enzymes to degrade proteins. Catalase is widely used in the process (Dey; Dora, 2014a; Silva, 2006; Gildberg; Stenberg, 2001), also of other enzymes such as chymotrypsin, trypsin, papain, pepsin (Silva, 2006), and proteases derived from

microorganisms (Dey; Dora, 2014b). The addition of enzymes that process can make it unviable, since the enzymatic extraction is an expensive process. To solve this problem, Cao et al. (2009) suggest to use the endogenous enzymes present in shrimp head as phosphorylases, lipases, and digestive enzymes.

Thus, the objective of this work was to develop a methodology for the production of a bioproduct (BP33, BP50, BP67) resulting from a mixture of protein hydrolysate, with variation in the concentration soybean meal and the hydrolysate. This product has great potential to replace fish meal in feed formulations for aquaculture.

5.2. Material and Methods

5.2.1. Hydrolysates

The heads of the shrimp *Litopenaeus vannamei* were obtained from a local fish processing industry (Noronha Pescados). The protein hydrolysate was prepared according to Cahu, et al. (2012). The heads were ground with distilled water (at concentration 1: 1), the liquid obtained was placed in a container in a water bath at 45 ± 2°C with constant agitation of 300 rpm to occur protein hydrolysis. After 120 minutes of hydrolysis the material was heated at 100°C for 10 minutes to stop hydrolysis. The compound was then filtered into a 1 mm sieve and the liquid obtained was frozen at -20°C.

5.2.2. Bioproducts

The protein hydrolysates were mixed with soy flour at concentrations of 33% (BP33), 50% (BP50) and 67% (BP67) hydrolysed. The mixtures were brought to 100°C for 4 hours, then were put on trays in an oven at 60°C for 48 hours. The dried flour were then ground in a bench grinder (IKA ® A11Básico, IKA ® Works, Inc., China) to reduce the particle size.

5.2.3. Chemical composition, amino acids and fatty acids

The analysis of the chemical composition, amino acid profile and fatty acids were made in CBO Analysis Laboratory. The following analyzes were performed: humidity (AOAC, 1996), crude protein (AOAC, 1995a), ether extract (UK, 1982), ash (AOAC, 1995b), carbohydrates, fiber, essential amino acids (White et al, 1986 and Hagen et al., 1989) and fatty acids (AOAC, 2007).

5.2.4. Chemical score of Bioproducts and Indispensable Amino Acid Index (IAAI)

Indispensable Amino Acid Index (IAAI) and chemical scores of bioproducts were calculated according to Hardy and Barrows (2002), relative to the EAA profile based on the reference amino acid in whole-egg protein. Indispensable Amino Acid Index (IAAI) is the ratio of the indispensable amino acid in the test protein (TP) divided by the indispensable amino acids in whole-egg protein (WEP) a follows:

$$IAAI = \frac{HIS(TP)}{HIST(WEP)} + \frac{ISO(TP)}{ISO(WEP)} + \frac{LEU(TP)}{LEU(WEP)} + \cdots + \frac{ARG(TP)}{ARG(WEP)} \times 100$$

Chemical scores were calculated using the following equation:

$$\text{Chemical score} = \frac{\text{EAA in test protein(g/kg)}}{\text{EAA amino acid in whole} - \text{egg protein(g/kg)}} \times 100$$

5.2.5. Crude Extracts

To obtain the tissue, the fish species *Oreochromis niloticus* (Nile tilapia), *Pseudoplatystoma coruscans* (pintado), *Astronotus ocellatus* (Oscar), *Cichla ocellaris* (tucunaré) and *Prochilodus costatus* (curimatã) obtained on the local market. A cut at the height of the lateral line was performed to remove the intestine and pyloric caeca or animal (depending on species). The preparation of crude extracts was performed according to the method described by Bezerra et al. (2001). The organs were weighed and homogenized with 15 mM saline at a ratio of 1:5 (W/V). The homogenates extracts

were then centrifuged at 10,000 xg for 15 minutes at 4°C, the supernatant was collected and stored at -20°C.

5.2.6. Crude extract protein quantification

Quantitation was performed using the BCA kit for proteins detection Thermo Scientific, according to the manufacturer's methodology.

5.2.7. Enzymatic activity

For total proteolytic activity was used azocasein substrate (1% in Tris-HCl 10 mM pH 8.0) at room temperature (25°C) using the methodology of Leighton et al. (1973) adapted for Alencar et al. (2003). The enzymatic reaction consisted of incubating 30 µL samples (crude extract of the intestine or caeca of fish species) with 50 µL of azocasein substrate (1%), being jointly carried out the reaction of white: 30 µL sample with 50 µL of buffer (10 mM Tris-HCl pH 8.0). The reaction lasted 60 minutes and was stopped with the addition of 240 µL of trichloroacetic acid (TCA) 10%. After addition of the acid to wait 15 minutes to stop the reaction, and then the samples were centrifuged at 8,000 xg for 5 minutes. After centrifugation, 70 µL of the supernatant was added to 130 µL of 1M NaOH in a microplate. The measurement of absorbance was performed in microplate reader at a wavelength of 450 nm.

5.2.8. Inhibitory effects of the ingredients

To evaluate the inhibitory effects adapted the methodology proposed by Lemos et al. (2004). The ingredients were dissolved for 16 hours in buffer (10 mM Tris-HCl pH 8.0) at a ratio of 1:12 on the magnetic stirrer, the contents were later centrifuged and the supernatant separated. In addition to the analyzes of the samples were made tests with positive control and negative control.

For the assays were incubated with 20µL ingredient dissolved and 10µL crude extract (for 90 minutes). To this mixture was added 50mL of 1% azocasein, pH 8.0. The negative control in parallel was made (dissolved ingredient (10µL) + crude extract

(20µL) + buffer (50mL)), a positive control (10µL of crude extract + 20µL buffer + 50 uL of azocasein) and white reaction: buffer (30µL) + 1% azocasein (50mL). The rest of the assay was performed according to the protocol activity with azocasein - Item 2.6. (Alencar et al., 2003). Inhibition was calculated as percentage of residual activity.

5.2.9. Data analysis

For data analysis were descriptive statistics used based on percentages through Excel 2007 and statistical inference with normality test (Kolmogorov-Smirnov), homogeneity of variances (Bartlett), analysis of variance (ANOVA) and Tukey's test, with significance level $P < 0.05$, using 7.7 Assistat and beta program Origin 8.0.

5.3. Results and Discussion

5.3.1. Chemical composition, amino acids and fatty acids

Percentages of protein, lipid, ash, fiber and other ingredients each bioproduct are shown in Table 1. The levels of nutrients proved quite close among bioproducts.

Burr et al (2012) farmed rainbow trout (*Oncorhynchus mykiss*) using a mixture of protein sources to replace fishmeal. Three different mixtures were used one with soy concentrate the other with barley concentrate and a last with higher levels of corn gluten. Beyond these ingredients all mixtures have led corn gluten, poultry meal, blood meal and soybean meal. All mixtures showed around 63% crude protein, and gluten meal with lipid mixture showed the highest value (4.6%). Even if all of these ingredients have been shown with a good level of crude protein, they needed amino acid supplementation (lysine, methionine, threonine, and taurine) according to the needs of the fish in question. Of all the mixtures used in the cultivation of rainbow trout, only composed of concentrated soy showed similar growth to control animals, the other two showed less weight gain.

When the ingredients of the aforementioned authors are compared to the present work it is noted that the amount of crude protein of bioproducts are at the same level and some have even more protein and the lipid levels were higher. The source plant

protein of the present work is the same as in the study Burr et al (2012) showed satisfactory growth of the animals.

For Nile tilapia commercial rations usually present fishmeal 10%. The availability of fishmeal is declining and its cost is growing investment in research for the replacement of this ingredient by other more sustainable has increased (Furuya et al, 2004a). The soybean meal has low costs and high levels of protein is an ingredient commonly used in replacement of fishmeal (El-Sayed, 1998). Furthermore, in salmonid and Nile tilapia farming it has been observed that the use of vegetable protein sources such as soy decrease the phosphorus excretion, thus reducing water pollution (Bergheim & Sveier, 1995; Furuya, 2004a). Even though with these advantages of soybean meal to be used with caution, because it has low availability of essential amino acids and various types of enzyme inhibitors (Krogdahl, Lea & Olli 1994; Furuya, 2004a).

The need to replace the fish meal by vegetal ingredients led to an increase in studies of fiber in the diet of fish (Fracalossi; Rodrigues; Gominho-Rosa, 2013). These fibers directly influence the intestinal functions which can be presented in animal feed as a main anti-nutritional factors (Hetland et al., 2004). High levels of fiber in the diet can increase the viscosity of the intestinal digesta, reducing the digestibility of protein and fat, slow intestinal transit and increase the moisture content of feces, factors that may cause a decrease in the animal growth (Storebakken, 1985; Leenhouwers et al., 2006; Rodrigues et al., 2012).

The maximum levels of fibers which can be included in the feed and which will not cause negative effects on the animal growth may vary from species to species. Amirkolaie et al. (2005) suggested that fiber levels for the Nile tilapia can not exceed 8%. Hansen and Storebakken (2007) were able to include up to 15% of fiber in the diet of rainbow trout (*Salmo gairdneri*) without loss on the grwoth performance and nutrient digestibility. However Hilton et al. (1983) observed a decrease in the growth of these trout fed levels between 10% and 20% of including fibers.

Some years ago the diets are based on crude protein needs of the fish being cultivated but at present these diets are formulated from the amino acid requirement of each species. Thus when protein sources do not respond to the demand of all the necessary amino acids ones that had disabled will be added to the diets industrially (Portz; Furuya, 2013).

All aminograms of bioproducts are shown in Table 2. In diets, amino acids are divided into two groups: essential and nonessential. Essential amino acids are not produced by the body the inclusion of these diet making it necessary. As for the nonessential produced by the body itself and is not therefore necessary to add these in diets (Buxbaum, 2007). For fish essential amino acids are arginine, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Halver; Delong; Mertz, 1957; Arai et al., 1980).

In rations where there is a replacement of animal to vegetable source protein, lysine generally shown as one of the most limiting amino acid in the production of such food (Mai et al., 2006). In some studies the reduction in lysine availability levels caused lower growth, smaller feed efficiency and health problems such as erosion on the dorsal and caudal fin (Ketola, 1983; Guillaume et al., 1999). Li et al. (2008) conducted experiments with varying levels of lysine and also demonstrated a direct interference of lysine in the growth and animal health.

In tilapia, *Oreochromis niloticus*, lysine levels should be between 5.5-6% of the total ration protein (Furuya et al., 2004b; Furuya et al., 2006; Takishita et al., 2009; Bomfim et al., 2010). For pacu, *Piaractus mesopotamicus*, the level of this amino acid should be between 4.7 to 5.7% crude protein (Bicudo et al., 2009; Abimorad et al., 2010) and tambaqui requirement shown around 7% (Oliveira; Miranda; Correia, 2013).

Another amino acid that may prove limiting is methionine, particularly in diets containing high percentages of soybean meal and peanut meal (Mai et al., 2006). Commonly this amino acid is found in conjunction with another amino acid, cystine which facilitates methionine synthesis can then get to spare 40-60% of methionine in the diets (Wilson, 2002). The requirement of these amino acids for pacu the varies between 1.6-1.7% (methionine+cystine) crude protein ration (Bicudo et al., 2009; Abimorad et al., 2010). For tilapia requirement of these two amino acids is around 3.2% and catfish (*Rhamdia quelen*) 4.3% total protein of the dietary offered (Santiago; Lovell, 1988; Montes Girao; Fracalossi, 2006).

Proteins, carbohydrates and lipids are molecules that are used as energy sources, lipids are macromolecules that have the highest caloric values releasing large amount of energy (Glencross, 2009). In fish, especially marine carnivores, the lipids appear as main energy source because its metabolize carbohydrates capacity is reduced. However

the species of herbivorous/omnivorous fish can metabolize complex carbohydrates and thus can also use them as an energy source (Watanabe, 1982; Smith, 1989).

Table 3 are the levels of fatty acids present in bioproducts. In general the levels of the fatty acids in bioproduct with lower concentrations of hydrolysate were lower levels fatty acids than those of two other bioproducts. The bioproducts the same concentration soy and hydrolysate and with a higher concentration of hydrolyzed and exhibited levels of fatty acids next.

Some authors have shown that the composition of fatty acids in the diet is directly connected the specific and nonspecific immunity (Montero et al., 2010; Xu et al., 2010; Zuo et al., 2012; Kiron et al., 2011; Sun et al., 2011; Li et al., 2013; Chen et al., 2016). Polyunsaturated fatty acids (PUFAs) of the n-6 and n-3 series operate regulators of cell signaling and gene expression and the most powerful modulators of cell membrane fluidity (Calder, 2006; Yaqoob and Calder, 2007).

5.3.2. Chemical score of Bioproducts and Indispensable Amino Acid Index (IAAI)

The chemical score of essential amino acids and and indispensable amino acids index of BP30, BP50, BP67, fish meal and soybean meal are shown in Table 4.

The nutritional value of the ingredients used in diets based on the composition of essential amino acids and the composition supplies the animal needs. Chemical score has been used to evaluate the nutritive value of a protein. This parameter compares levels of essential amino acids between the test and standard proteins (Bhaskar et al., 2006). Values next to 100% is considered of high nutritional value (Silva et al, 2014).

Silva et al (2014) produced fish hydrolysates and found chemical score values greater than those of the present work however all the essential amino acids of bioproducts presented satisfactory levels which shows that the three ingredients can be used in the formulation well balanced diets.

5.3.3. Protein quantification and total proteolytic activity assay

The protein quantification results and proteolytic activity of crude extracts from the gut of *Oreochromis niloticus* (Nile tilapia), *Pseudoplatystoma coruscans* (pintado),

Astronotus ocellatus (oscar) and *Cichla ocellaris* (tucunaré) and pyloric caeca of *Prochilodus costatus* (curimatã) they are contained in Table 5.

The proteolytic activities were statistically different for all species analyzed, the species with the highest activity was the Oscar and the lowest activity tucunaré. But both species had a much lower value than found by Soares et al. (2008), in tucunaré analysis the authors found endogenous activity of alkaline proteases of 61.2 ± 4.8 and Cavero (2004) found 74.21 ± 4.52 for those same enzymes. The difference may be related to feeding species (Sabapathy & Teo, 1993), in addition to age and eating habits that can interfere with the enzymatic activity values.

Omnivorous fish usually have a very diverse enzymatic arsenal usually with high values for most enzymes. However the Nile tilapia did not show as significant values corroborating the hypothesis that the power used for these fish could be out of the nutritional requirements of the species.

5.3.4. Inhibitory effects of the ingredients

The effects inhibition of the ingredients over crude extract proteases of the digestive tract fish studied are shown in table 6. For all species studied soybean meal had the highest inhibition rate, this can be explained by the fact that soy include peptides that bind to the enzymes chymotrypsin, trypsin and carboxypeptidase, leaving them inactive (Pike; Hardy, 1997; Silva; Silva, 2000).

Soybean meal came to present a residual activity of 27.4 ± 6.8% for Nile tilapia (species had greater inhibition of proteases in the presence of soybean). The tucunare despite being a carnivore showed a reasonable residual activity to the feed (70.6 ± 11.3%). This fact is very interesting for aquaculture as it could reduce costs in the production of animal food for this species. Already pintado showed no similarity to the tucunaré in relation to soybean meal, in this case the residual proteolytic activity was 42.9 ± 4.2%, suggesting that this species has increased demand for animal protein. Soybean inhibitors action is still observed in the cutivation of other aquatic organisms, as observed in studies in *Litopenaeus vannamei* and *Litopenaeus californiensis* (García-Carreño et al., 1997).

The fish meal has good nutritional values for the supply and absorption of protein and amino acids (Pezzato et al., 1997). Thus it is expected that the residual activity values of crude extracts tested are higher for this ingredient. All species showed good activity values to be subjected to fishmeal, especially omnivorous. This denotes an important input in the production of animal food. But is not a sustainable response and its use in the manufacture of feed for other animals has increased the cost (Mendoza et al., 2001; Martins, 2011;. Faria et al, 2001). Thus replacing the alternative food fish meal are being tested in the search for cost reduction and the increase in nutrients gains (Boscolo et al, 2011; Signor et al., 2007).

The bioproducts tested were satisfactory compared to the soybean meal and fish meal. Their residual enzyme activities were mostly higher than those observed for soybean meal, and similar in some cases fish meal. The hydrolysate showed minimal inhibition for Nile tilapia with a residual activity of 94.9 ± 9.5%, BP33, BP50, BP67, showed low inhibition, 70.5 ± 8.1%; 92.1 ± 2.4% and 83.6 ± 6.0%, respectively. For pintado, hydrolyte, BP33, BP50 and BP67 showed low inhibition (residual activity of 70.3 ± 6.0%, 77.2% ± 1.8, 88.2 ± 2.4% and 93.2% ± 1.6, respectively). The hydrolysate was presented as an excellent choice for the Oscar as the result (99.8 ± 3.6%) is close to the maximum, the BP33 products, BP50 and BP120 also showed good values, 84.5 ± 7 0%; 113.4 ± 4.9% and 82.4 ± 5.3%, respectively. The residual proteolytic activity of the tucunaré to the hydrolysate was 117.2 ± 13.4%, these values indicate great action of enzymes. Presented to the satisfaction of the BP33 (79.4 ± 21.4%), BP50 (74.9 ± 1.2%), and BP67 (69.6 ± 2.6%). The Curimatã is species detritivore also showed good acceptance of bioproducts, with residual activity for the hydrolysate of 87.9 ± 7.2%. The bioproducts BP33, BP50 and BP67 also showed good values, 75.5 ± 3.3%; 70.0 ± 8.1% and 73.7 ± 5.8%, respectively.

Analyzing inhibition species was observed that Nile tilapia was lower with use of the hydrolysate (94.9 ± 9.5%) without the addition of vegetable sources. Oscar the better option was the bioproduct BP50 (113.4 ± 4.9%) with addition of 50% of vegetable protein sources. For pintado the most satisfactory result was with the supply of the bioproduct BP67 (93.2 ± 1.6%). Regarding the tucunaré the best results were also in the absence of sources of vegetable origin the hydrolysate showing 117,2 ± 13,4%

residual activity. And curimatã also showed higher residual activity with hydrolysate (87.9 ± 7.2%).

Although most species have been better activity in the presence of hydrolysate, the use of other bioproducts noteworthy, due to higher than soybean meal and similar to fishmeal. The bioproducts shown to be an excellent alternative for the feed industry, since for obtaining them are used waste from the own fishing industry increased of vegetable protein, making these more cost effective protein sources, since it had low inhibition rate of proteases and a good amount of crude protein. Even though studies in some species of fish have satisfactory growth using protein sources of vegetable origin it is important to note that the bioproduct even using soybean showed lower inhibition of digestive tract proteases which can be attributed to inactivation of these inhibitors of this preparation methodology ingredient.

5.4. Conclusions

The products presented here show with excellent protein levels, fatty acids and fiber. The residual proteolytic activity of several species of fish have shown that possessed low bioproducts proteolytic inhibition. Even if the soybean when in contact with enzymes in the digestive tract of fish inhibits the enzymatic activity after the preparation of the bioproduct enzyme inhibition experiments showed that these inhibitors have been inactivated. The protein hydrolysate besides showing an excellent source when mixed with soy in a product with optimal essential amino acid profile feeding aquatic animals.

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Table 1: Chemical composition of BP33, BP50, BP67 (%).

Nutrient	**Bioproduct**		
	BP33	**BP50**	**BP67**
Protein	58,04	60,51	64,73
Ether Extract	7,65	10,29	10,45
Fiber	3,89	2,92	2,40
Ash	8,35	9,24	9,69
Other nutrients	22,07	17,04	12,73

Table 2: Essentials and nonessentials amino acids composition (%) of the BP33, BP50, BP67 and other ingredients used in feeds for aquatic organism. (DM basis)

Amino acids	BP33	BP50	BP67	Fish Meal	Soybean Meal
Essentials					
Phenylalanine	2,60	2,63	2,76	1,81	1,91
Valine	2,64	2,83	3,02	2,59	1,90
Arginine	4,38	4,57	4,84	3,78	3,05
Thryptophan	0,51	0,56	0,57	0,39	0,80
Threonine	1,97	2,10	2,22	1,99	1,51
Leucine	4,21	2,23	4,55	3,77	2,91
Histidine	1,39	1,40	1,48	1,40	1,25
Isoleucine	2,15	2,23	2,36	2,15	1,75
Methionine	0,70	0,98	1,08	1,80	0,47
Lysine	3,92	4,10	4,47	3,98	2,57
AAE	24,47	23,63	27,35	23,66	18,12
Nonessentials					
Cystine	1,13	1,22	1,32	-	-
Aspartic Acid	6,93	6,78	6,97	-	-
Glutamic Acid	10,27	10,05	10,48	-	-
Proline	3,44	3,77	4,23	-	-
Tyrosine	2,04	2,11	2,21	-	-
Serine	2,80	2,71	2,81	-	-
Alanine	3,51	3,96	4,48	-	-

(-) Data not provide by the authors.

AAE: total essential amino acids

[a]Halver (1995)

Table 3: Percentages fatty acids in bioproducts 33%, 50% and 67%.

Fatty acids	**Bioproduct**		
	BP33	**BP50**	**BP120**
Butyric Acid (C4)	0,02	NC	0,03
Lauric Acid (C12)	0,0	NC	0,0
Myristic Acid (C14:0)	0,03	0,05	0,05
Pentadecanoic Acid(C15:0)	0,01	0,02	0,02
Palmitic Acid (C16:0)	1,41	1,95	1,96
Margaric Acid (C17:0)	0,03	0,05	0,05
Estearic Acid (C18:0)	0,44	0,64	0,64
Arachidic Acid (C20:0)	0,02	0,02	0,02
Heneicosanoic acid(C21:0)	0,01	0,01	0,01
Behenic Acid (C22:0)	0,03	0,02	0,02
Tricosanoic (C23:0)	0,01	0,01	0,01
Lignoceric Acid (C24:0)	0,01	0,01	0,01
Saturated Fat	2,01	2,78	2,83
Palmitoleic Acid (C16:1)	0,08	0,13	0,13
Oleic Acid (C18:1n9c)	1,43	2,06	2,11
Cis-Eicosenoic Acid (C20:1)	0,06	0,11	0,11
Erucic Acid (C22:1n9)	0,01	0,02	0,02
Nervonic Acid (C24:1)	0,02	0,02	0,02
Monounsaturated	1,62	2,36	2,42
Linoleic Acid (C18:2n6)	2,33	2,58	2,51

Gamma Linolenic Acid (C18:3n6)	0,00	0,01	0,01
Linolenic Acid (C18:3n3)	0,23	0,22	0,20
Cis-Eicosadienoic Acid (C20:2)	0,12	0,20	0,21
Cis-Eicosatrienoic Acid (C20-3n3)	0,02	0,03	0,03
Cis-Eicosatrienoic Acid (C20:3n6)	0,02	0,03	0,03
Arachidonic Acid (C20:4n6)	0,11	0,18	0,19
Docosadienoic Acid (C22:2n6)	0,00	0,00	0,0
Cis-Eicosapentaenoic Acid (C20:5n3)	0,26	0,43	0,45
Cis-Docosahexaenoic Acid (C22:6n3)	0,92	1,48	1,58
Polyunsaturated Fat	4,02	5,14	5,20
Elaidic Acid (C18:1n9t)	0,01	0,01	0,01
Trans Fat	0,01	0,01	0,01
Unsaturated Fats	5,64	7,51	7,62

Table 4: Indispensable Amino Acid Index (IAAI) and chemical score of amino acids of BP33, BP50 and BP67 compared with fish meal and soybean meal. (DM basis)

Amino acids	**Chemical scores = limiting amino acid in test protein/amino acid in whole-egg protein (%)**					**Whole egg amino acids (g/kg)**
	BP33	**BP50**	**BP67**	**Fishmeal[a]**	**Soybean Meal[a]**	
Phenylalanine	41,27	41,75	43,81	28,73	30,32	63,0
Valine	36,67	39,31	41,94	35,97	26,39	72,0
Arginine	66,36	69,24	73,33	57,27	46,21	66,0
Thryptophan	34,00	37,33	38,00	26,00	53,33	15,0
Threonine	45,81	48,84	51,63	46,28	35,12	43,0
Leucine	45,76	24,24	49,46	40,98	31,63	92,0
Histidine	57,92	58,33	61,67	58,33	52,08	24,0
Isoleucine	27,92	28,96	30,65	27,92	22,73	77,0
Methionine	17,50	24,50	27,00	45,00	11,75	40,0
Lysine	56,00	58,57	63,86	56,86	36,71	70,0
IAAI[b]	429,21	431,07	481,34	423,34	346,27	-

[a]Halver (1995).

[b]According to Hardy and Barrows (2002).

Table 5: Protein quantification and proteolytic activity of crude extracts *Oreochromis niloticus*, *Prochilodus costatus*, *Pseudoplatystoma coruscans*, *Astronotus ocellatus* and *Cichla ocellaris*.

Species	**Protein quantification (mg · mL^{-1})**	**Proteolytic activity* (U · mg Proteína^{-1})**
Nile tilapia (*O. niloticus*)	4,11 ±1,7^{a}	1,66 ± 0,08^{d}
Curimatã (*P. costatus)*	4,10 ±1,52^{a}	1,89 ± 0,20^{b}
Pintado (*P. coruscans)*	3,51 ±1,07^{ab}	1,85 ± 0,09^{c}
Oscar (*A. ocellatus)*	4,203 ±1,6^{a}	1,92 ± 0,08^{a}
Tucunaré (*C. ocellaris*)	2,12 ±1,04^{b}	1,29 ± 0,25^{e}

*Substrate - Azocasein 1.0% pH 8.0.

Different letters overwritten in the same column indicate statistical difference (P <0.05) by Tukey test. Values are mean ± standard deviation of three replicates.

Table 6: Residual proteolytic activity (%) present in the digestive tract of crude extracts of Nile tilapia, pintado, oscar, tucunaré and curimatã under the influence of ingredients that may be used in the feed industry.

Ingredients	**Residual proteolytic activity (%)***				
	Nile tilapia	**Pintado**	**Oscar**	**Tucunaré**	**Curimatã**
Soybean meal	27,4 ± 6,8	42,9 ± 4,2	45,2 ± 7,3	70,6±11,3	45,2 ± 3,2
Fish meal	100,7 ± 10,1	89,6 ± 11,8	115,2 ± 9,7	101,9 ± 14,4	89,9 ± 0,2
Hydrolysate	94,9 ± 9,5	70,3 ± 6,0	99,8 ± 3,6	117,2 ± 13,4	87,9 ± 7,2
BP 33	70,5 ± 8,1	77,2 ± 1,8	84,5 ± 7,0	79,4 ± 21,4	75,5 ± 3,3
BP 50	92,1 ± 2,4	88,2 ± 7,0	113,4 ± 4,9	74,9 ± 1,2	70,0 ± 8,1
BP 67	83,6 ± 6,0	93,2 ± 1,6	82,4 ± 5,3	69,6 ± 2,6	73,7 ± 5,8

6. CAPÍTULO II

Os resultados do presente trabalho estão apresentados no artigo intitulado "**Biological value of shrimp protein hydrolisates with different time of hydrolis and its pottential application in tropical fish feed**" (manuscrito) e serão submetidos ao periódico LWT- Food Science and Technology. (ISSN: 0023-6438)

Biological value of shrimp protein hydrolisates with different time of hydrolis and its pottential application in tropical fish feed

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Abstract

The feed used in aquaculture need high protein levels and a good amino acid profile. The degree of hydrolysis of the protein source in diets influences directly the quality of provided protein. This work aims at the production of two bioproducts based on hydrolysates with times of 30 and 120 minutes of hydrolysis. The values of the chemical composition demonstrated levels of proteins, lipids, ash, fiber and others components very close between both bioproducts. The chemical score values showed that the amino acid level in bioproducts is demonstrated with ideal values for use in balanced rations. The absence of specific microorganisms based on the requirement of Brazilian Health Surveillance Agency (ANVISA) demonstrates the good microbiological quality of the products. The mesophilic analysis showed that the bioproducts can be stored up to four and a half months at room temperature without risk to health. These data can support industrial uses of the bioproducts, which requires increased shelf stability time and resistance to microbial spoilage**.**

Key-words: Protein hydrolysate; bioproduct; extended shelf life.

6.1. Introduction

Waste shrimps are a rich source of amino acids, peptides, protein and other useful biochemicals which may be recovered for utilization in various applications, including animal feed (Simpson, 1998). It is estimated that 52% of total shrimp production originates byproducts - head, shell and tail (Heu et al., 2003).

Aquaculture intensification and others ecological issues directly affect fish species populations used to produce fish meal. Thus, this kind of flour has became expensive and a ecologically unfriendly choice. This situation raised a demand for new adequate proteins sources to amino acids requirements and, at the same time, a search to reduce production costs and maximizes fish production and minimize ecological impacts caused by culture systems (Cyrino et al., 2010).

The feed used in aquaculture need high protein levels and a good amino acid profile. In general, protein sources like blood meal, soybeam meal and peanut meal, don't have adequate nutritional values and essential amino acids profile similar to fish meal (Portz, Cyrino, 2003; Leal et al., 2010).

Protein hydrolysis is the process whereby peptide bonds of the proteins are dug peptides yielding, this process can be performed by acids, bases and enzymes. Enzymatic hydrolysis, in addition to being more efficient than chemical hydrolysis, has larger application in food products (Kristinsson & Rasco, 2000; T onon et al., 2016). Usually the shrimp processing by-products are submitted to enzymatic hydrolysis with proteases such as alcalase, papain, pepsin, chymotrypsin or trypsin (Chalamaiah et al, 2012). The protein hydrolysis by-products using selected proteolytic enzymes allows a certain control of the products formed. The use of appropriate concentrations of enzyme and knowledge of reaction times allow production of hydrolyzed compounds of different molecular structures and thus different functional properties that can find applications in various food formulations (Santos et al., 2009).

Proteolytic enzymes use, however, limits the production of these byproducts shrimp head because the process more expensive. One way around this difficulty is the use of endogenous enzymes present in the head of shrimp (phosphorylases and lipases) and hepatopancreas (digestive enzymes) (Cao et al., 2009).

The nutritional quality of these hydrolysates is related to their content of low molecular weight peptides, especially di- and tripeptides these are absorbed faster than free amino acids and proteins (Silvestre; Hamon e Yvon, 1994; Shimamura et al., 1999; Boza, 2000).

The hydrolysis time used is of utmost importance for the peptide composition of the protein hydrolysate. Thus, this paper aims to produce two bioproducts (hydrolysate and vegetal protein), potential use in aquaculture, with different hydrolysis times (BP30 and BP120).

6.2. Material and Methods

6.2.1. Hydrolysates

The heads of the shrimp *Litopenaeus vannamei* were obtained from a local fish processing industry (Noronha Pescados LTDA, Recife, PE - Brazil). The protein hydrolysate was prepared according to Cahu, et al. (2012). The heads were ground with distilled water (1:1 p/v), the liquid obtained was placed in a container in a water bath at 45 ±2°C with constant agitation of 300 rpm to occur protein hydrolysis. Two treatments were performed in the first hydrolysis treatment was carried out for 30 minutes and the second hydrolysis lasted 120 min.

After 30 minutes and 120 minutes hydrolysis of the material was heated at 100°C for 10 minutes to stop hydrolysis. The compound was then filtered into a 1 mm sieve and the liquid obtained was frozen at -20°C.

6.2.2. Bioproducts

The protein hydrolysates were mixed with soy flour at concentrations of 67% hydrolysed and 33% soy.The mixtures were brought to 100°C for 4 hours, then were put on trays in an oven at 60°C for 48 hours. The dried flour were then ground in a bench grinder (IKA ® A11Básico, IKA ® Works, Inc., China) to reduce the particle size.

6.2.3. Chemical composition, amino acids and fatty acids

The analysis of the chemical composition, amino acid profile and fatty acids were made in CBO Analysis Laboratory. The following analyzes were performed: humidity (AOAC, 1996), crude protein (AOAC, 1995a), ether extract (UK, 1982), ash (AOAC, 1995b), carbohydrates, fiber, essential amino acids (White et al, 1986 and Hagen et al., 1989) and unsaturated fatty acids (AOAC, 2007).

6.2.4. Chemical score of Bioproducts and Indispensable Amino Acid Index (IAAI)

Indispensable Amino Acid Index (IAAI) and chemical scores of bioproducts were calculated according to Hardy and Barrows (2002), relative to the EAA profile based on the reference amino acid in whole-egg protein. Indispensable Amino Acid Index (IAAI) is the ratio of the indispensable amino acid in the test protein (TP) divided by the indispensable amino acids in whole-egg protein (WEP) a follows:

$$IAAI = \frac{HIS(TP)}{HIST(WEP)} + \frac{ISO(TP)}{ISO(WEP)} + \frac{LEU(TP)}{LEU(WEP)} + \cdots + \frac{ARG(TP)}{ARG(WEP)} \times 100$$

Chemical scores were calculated using the following equation:

$$\text{Chemical score} = \frac{\text{EAA in test protein(g/kg)}}{\text{EAA amino acid in whole} - \text{egg protein(g/kg)}} \times 100$$

6.2.5. Shelf-life evaluation

6.2.5.1. Specific Microorganisms

Microbiological analysis (molds and yeasts, Coliforms at 45ºC, Coagulase positive Staphylococci, Salmonella and Pseudomonas spp) of bioproducts (BP30 and BP120) were performed to certify microbiological contamination at time 0 and at the end of production time. Analyses were performed in Experiment Laboratory and Food Analysis (LEAAL) of the Nutrition Department at the Federal University of Pernambuco.

6.2.5.2. Total count of mesophilic microorganisms

The total count of mesophilic in bioproducts (BP30 and BP120) were performed every 15 days. 5 mg bioproduct samples were dissolved in 5 ml of peptone water and homogenized under sterile conditions in biological safety cabinet Pachane (PA 410). They were collected 250 µL and 500 µL each sample and inoculated on 2 Petri dishes. To each plate was added 10 ml of Plate Count Agar (PCA - Standard Methods Agar Acumedia). The samples were homogenized immediately after being poured into the medium by rotations of the Petri dishes to obtain uniform dispersion of colonies. After complete solidification the plates are inverted and incubated for 2 days at 37°C for mesophilic microorganisms count.

6.3. Results and Discussion

6.3.1. Chemical composition, amino acids and fatty acids of Bioproducts

Chemical composition analyses of bioproducts are in Table 1. Next values of proteins, lipids, ash, fibers and others components were found in the composition of bioproducts.

The demand for ingredients that can replace fishmeal in the feed in aquaculture has taken a lot of strength in recent years. Sun et al. (2015) proposed a cotton seed meal fermented for feeding *Acanthopagrus schlegelii*. In their work they compared feeding of fish meal, cotton seed flour and fermented cotton seed meal. Fishmeal they used had levels of 64.8% protein, 4% lipid and 11.1% ash, alternative levels of protein ingredients showed 47.1% and 48.8% for the cotton seed flour and for cotton seeds flour fermented, respectively. The content of lipids showed near values 1.4 and 1.5% for cotton seeds flour and cotton seed flour fermented, respectively.

Hydrolysates of tilapia carcasses show average levels of 52% protein, values below the bioproduct studied (Silva et al, 2014). However a protein level close to this work was obtained in a viscera hydrolysate Persian sturgeon (*Acipenser persicus*) with exogenous enzyme (Ovissiopour et al, 2009).

The compositions values of essential and nonessential amino acids for fish found bioproducts, fish meal and soybean meal are shown in Table 2. Glutamic acid, aspartic acid and arginine amino acids were highest expression in both bioproducts. These amino acids were also more expressive in shrimp hydrolysate (Tonon et al., 2016) and to hydrolysates obtained from the blue shark skin (Rodríguez-Díaz et al., 2011). Hydrolysates of Nile perch (*Lates niloticus*), grass carp (*Ctenopharyngodon idella*) and Nile tilapia (*Oreochromis niloticus*) showed a higher content of glycine and alanine (Wasswa, Tang & Gu, 2008).

Proteolytic hydrolysis is a process used to increase the nutritional value of proteins by improving their digestibility (Li et al., 2010), as the time of hydrolysis will influence the size of the formed peptides, some studies with shrimp hydrolysates showed that hydrolysis has two stages. The first stage reaction speed maximum was shown as the high molecular weight proteins showed intense degradation of the peptide bonds. The second stage begins with a one hour reaction where there is a decrease in rate which reaches the stationary behavior in six hours. This reduction reaction rate can be explained by the reduced amount of peptide bonds breaking available for possible inhibition by products formed during the reaction and the enzyme autolysis process (González-Tello et al. 1994; Guerard, Guimas, & Binet, 2002, Tonon et al., 2016).

Table 3 shows the composition of fatty acids of bioproducts. Essential fatty acids are those that are not produced by the body itself, these must be included in the diet. These essential fatty acids are divided into two groups: derivatives of linoleic acid - omega-6 group (n-6); and derivatives linolenic acid - omega-3 group (n-3) (Calder, 2012). All these fatty acids are considered essential present in the composition of bioproducts.

According to Sargent et al. (2002) fatty acids: C16:0, C18:1 n-9, C20:1 n-9, C22:1 n-9, C20:5 n-3 (EPA) and C22:6 n-3 (DHA), are the main fish used as the energy source for development, growth and fish swimming in addition to the reproductive process. For Atlantic salmon (*Salmo salar*) there is a positive correlation between the levels of fatty acids with 18 carbons in the muscles, especially C18:1 n-9 and C18:2 n-6 (LA), and maximum swimming speed these animals (McKenzie et al, 1998).

In addition to the energy function, lipid form the cell membranes. In fish, fatty acids forming the phospholipid molecules are mainly 16:0, 18:1 n-9, C20:5 n-3 (EPA)

and C22:6 n-3 (DHA). In these organisms the DHA is the main component of cell membranes of the brain and retina cell (Sargent, 2002). Diets with a low level of this fatty acid decreased visual capacity of herring larvae (*Clupea harengus*) and the total size of the brain and the regions responsible for vision and swimming larvae of *Seriola quinqueradiata* ("yellowtail"), and make them dispersed without the ability to form schools (Bell et al, 1995; Ishizaki et al, 2001).

6.3.2. Chemical score of Bioproducts and Indispensable Amino Acid Index (IAAI)

The chemical score and indispensable amino acids index of BP30, BP120, fish meal and soybean meal are in table 4. From the essential amino acid levels, indispensable amino acid and index (IAAI) and chemical score is usually used to evaluate the nutritive value of a protein source. This parameter compares the amino acids in a test protein with those in whole-egg protein (Hardy and Barrows, 2002) and values next to 100% is considered of high nutritional value.

All chemical scores of bioproducts presented higher values than fish meal and soybean meal however Silva et al. (2014) they found higher values for their fish hydrolysates. Fish meal demonstrated that tryptophan and isoleucine are limiting amino acids for soybean meal were the limiting amino acids isoleucine, valine and methionine. For all these amino acids the chemical score showed appropriate for bioproducts. All these values show that both bioproducts have good nutritional levels and can be used as protein sources in fish feed.

6.3.3. Shelf-life evaluation

6.3.3.1. Specific Microorganisms

The analyzes of specific microorganisms in the beginning and end of shelf-life experiment are present in Tables 5 and 6. The Brazilian Health Surveillance Agency (ANVISA) establishes health microbiological standards for food and determines the criteria for completion and interpretation of results of microbiological analysis of food for human consumption. As tolerance limit for indicative sample fish fresh, chilled or

frozen not eaten raw, fixed the following amounts: 103 CFU/g for Staphylococcus positive coagulase, 102 MPN/g Coliform and absence of Salmonella sp, the latter being classified as danger of moderate severity (Silva & Filho, 1999). The maximum limit for heterotrophic bacteria aerobic psychrotrophic (BHAP) for seafood is 107 CFU/g (ICMSF, 1986). All values are found within the required standard.

In fish processing industry, hygiene procedures are essential to ensure product quality. Thus, the use of rigorous hygiene, following appropriate standards, favors the quality control, enables production costs, satisfy consumers and protect them against risks to their health and to respect the rules and microbiological standards recommended by law (Germano & Germano, 2001).

The Salmonella is a patogenic agent that can stand for long periods in various types of materials. These microorganism can therefore pose a hazard in animal feed. Even though seafood derivatives are not very susceptible to Salmonella but some strains seem able to adapt to this type of substrate. Many studies are conducted to reduce contamination of foods or ingredients of these foods in the factory (Vestby et al., 2009; Jones, 2011; Pastore et al, 2013).

In this study the presence of Salmonella was not detected in both bioproducts. Samples collected by Jones (2011) in a fish meal factory were negative for the presence of Salmonella too.

Tabib, Jones and Hamilton (1981) analyzed ingredients for poultry feed and found to fish meal and soybean meal values of molds and coliforms much higher than found for the bioproducts. Coliforms were on average 50 units per gram for two ingredients and the mold presented a mean of 100 units to the soybean meal and 200 for fishmeal.

6.3.3.2. Total count of mesophilic microorganisms

The count of mesophilic micro-organisms is presented in Table 7. The bioproducts had their storage time of five months.

Most of the pathogenic micro-organisms and is mainly represented by mesophilic bacteria and molds. All micro-organisms that are a risk to food safety

multiply ideally located in the middle range of 30°C to 45°C (Germano, Germano & Ungar, 2003).

Bueno-Solano et al., (2009) reported total mesophilic of 1730 cfu in shrimp protein hydrolysate and this number does not represent a health risk. This value is much higher than that found in 125 days of bioproduct stored at ambient temperature. Gonçalves and Viegas (2007) had 120 days of shrimp silage stability from measurements of the Chemical composition and non-protein nitrogen levels.

6.4. Conclusion

Chemical composition analysis of bioproducts using hydrolysates with different hydrolysis times no showed significant difference in the levels and composition of amino acids and fatty acids. Microbiological analysis both bioproducts were within required levels and proved microbiologically stable for five months.

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Table 1: Chemical composition of the bioproducts. (DM basis)

Nutrient	Bioproduct	
	BP30 (%)	**BP120 (%)**
Protein	66,11	64,73
Ether extract	6,77	10,45
Fiber	2,49	2,40
Ash (%)	11,18	9,69
Others (%)	13,45	12,73

Table 2: Essentials and nonessentials amino acids composition (%) of the bioproducts and other ingredients used in feeds for aquatic organism. (DM basis)

Amino acids	**BP30**	**BP120**	**Fish meal[a]**	**Soybean Meal[a]**
Essentials (%)				
Phenylalanine	2,84	2,76	1,81	1,91
Valine	2,90	3,02	2,59	1,90
Arginine	5,49	4,84	3,78	3,05
Thryptophan	0,57	0,57	0,39	0,80
Threonine	2,62	2,22	1,99	1,51
Leucine	4,67	4,55	3,77	2,91
Histidine	1,55	1,48	1,40	1,25
Isoleucine	2,49	2,36	2,15	1,75
Methionine	1,45	1,08	1,80	0,47
Lysine	4,03	4,47	3,98	2,57
AAE	28,61	27,35	23,66	18,12
Nonessentials (%)				
Cystine	0,74	1,32	-	-
Aspartic Acid	6,55	6,97	-	-
Glutamic Acid	10,28	10,48	-	-
Proline	4,62	4,23	-	-
Tyrosine	2,30	2,21	-	-
Serine	3,13	2,81	-	-
Alanine	4,21	4,48	-	-

(-) Data not provide by the authors.

AAE: total essential amino acids

[a]Halver (1995)

Table 3: Fatty acids composition of bioproducts (%). (DM basis)

Fatty acids	**Bioproducts**	
	BP30	**BP120**
Butyric Acid (C4)	ND	0,03
Lauric Acid (C12)	ND	0,0
Myristic Acid (C14:0)	0,02	0,05
Pentadecanoic Acid(C15:0)	0,02	0,02
Palmitic Acid (C16:0)	1,17	1,96
Margaric Acid (C17:0)	0,03	0,05
Estearic Acid (C18:0)	0,44	0,64
Arachidic Acid (C20:0)	0,01	0,02
Heneicosanoic acid(C21:0)	ND	0,01
Behenic Acid (C22:0)	0,01	0,02
Tricosanoic (C23:0)	0,01	0,01
Lignoceric Acid (C24:0)	0,02	0,01
Saturated Fat	1,73	2,83
Palmitoleic Acid (C16:1)	0,07	0,13
Oleic Acid (C18:1n9c)	1,31	2,11
Cis-Eicosenoic Acid (C20:1)	0,05	0,11
Erucic Acid (C22:1n9)	ND	0,02
Nervonic Acid (C24:1)	0,01	0,02
Monounsaturated	1,44	2,42
Linoleic Acid (C18:2n6)	2,03	2,51
Gamma Linolenic Acid (C18:3n6)	ND	0,01
Linolenic Acid (C18:3n3)	0,15	0,20
Cis-Eicosadienoic Acid (C20:2)	0,01	0,21
Cis-Eicosatrienoic Acid (C20-3n3)	0,01	0,03
Cis-Eicosatrienoic Acid (C20:3n6)	0,01	0,03
Arachidonic Acid (C20:4n6)	0,14	0,19
Docosadienoic Acid (C22:2n6)	ND	0,0
Cis-Eicosapentaenoic Acid (C20:5n3)	ND	0,45
Cis-Docosahexaenoic Acid (C22:6n3)	0,45	1,58
Polyunsaturated Fat	ND	5,20
Elaidic Acid (C18:1n9t)	ND	0,01
Trans Fat	ND	0,01

Unsaturated Fats	ND	7,62

ND - not detected

Table 4: Indispensable Amino Acid Index (IAAI) and chemical score of amino acids of bioproducts compared with fish meal and soybean meal. (DM basis)

Amino acids	**Chemical scores = limiting amino acid in test protein/amino acid in whole-egg protein (%)**				**Whole egg amino acids (g/kg)**
	BP30	**BP120**	**Fish meal[a]**	**Soybean meal[a]**	
Phenylalanine	45,08	43,81	28,73	30,32	63,0
Valine	40,28	41,94	35,97	26,39	72,0
Arginine	83,18	73,33	57,27	46,21	66,0
Thryptophan	38,00	38,00	26,00	53,33	15,0
Threonine	60,93	51,63	46,28	35,12	43,0
Leucine	50,76	49,46	40,98	31,63	92,0
Histidine	64,58	61,67	58,33	52,08	24,0
Isoleucine	32,34	30,65	27,92	22,73	77,0
Methionine	36,25	27,00	45,00	11,75	40,0
Lysine	57,57	63,86	56,86	36,71	70,0
IAAI[b]	508,97	481,34	423,34	346,27	-

[a]Halver (1995).

[b]According to Hardy and Barrows (2002).

Table 5: Specific microorganisms analysis of the products on the first day of production.

Bioproducts	**Coliforms 45 °C MPN/g**	**Coagulase Positive *Staphylococcus* CFU/g**	***Salmonella spp***	**Yeasts and molds CFU/g**	***Pseudomonas aeruginosa***
BP30	< 3.0	< 10	Absence	< 10	< 1.1
BP120	< 3.0	< 10	Absence	< 10	< 1.1

MPN = Most Probable Number

CFU = colony forming unit

Table 6: Specific microorganisms analysis of the products on the last day experiment of Shelf-life.

Bioproducts	**Coliforms 45 °C MPN/g**	**Coagulase Positive *Staphylococcus* CFU/g**	***Salmonella spp***	**Yeasts and molds CFU/g**	***Pseudomonas aeruginosa***
BP30	< 3.0	< 10	Absence	< 10	< 1.1
BP120	< 3.0	< 10	Absence	< 10	< 1.1

Table 7: Total count of mesophilic microorganisms for five months of storage bioproducts at ambient temperature.

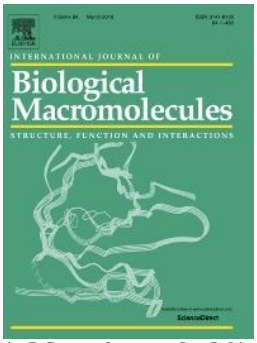
	Estorage days										
Bioproducts	**Mesophilic (CFU/g)**										
Days	**0**	**15**	**30**	**45**	**60**	**75**	**90**	**95**	**110**	**125**	**140**
BP30	ND	ND	ND	ND	ND	ND	ND	ND	ND	$1.0x10^2$	Uncountable
BP120	ND	ND	ND	ND	ND	ND	ND	ND	ND	$1.3x10^2$	Uncountable

CFU = colony forming unit/g

ND - not detected

7. CAPÍTULO III

Esse artigo intitulado "**Bioactive molecules from Malaysia giant prawn (*Macrobrachium rosenbergii*) processing waste**" (manuscrito) será submetido à revista "International Journal of Biological Macromolecules"



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Bioactive molecules from Malaysia giant prawn (*Macrobrachium rosenbergii*) processing waste

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Abstract

Shrimp processing wastes are recognized sources of bioactive molecules that are recovered and have many potential applications. The freshwater prawn *Macrobrachium rosenbergii*, also known as giant Malaysia prawn, a species of shrimp native to the Indo-pacific region, has been farmed by small local producers in Northeastern Brazil, due to its resistance and easily adaptation to the climate conditions. The good fillet quality is also a attractive characteristic of *M. rosenbergii*, while its non-food by-products (cephalotorax, carapace, pereiopods and tail) can constitute approximately 50% of the whole animal. These residues can be used as sources of biomolecules, as value added products apart of the main commercial product. In this work, we analyzed the recovery of protein, chitin, chitosan and carotenoids from *M. rosenbergii* heads submitted to autolysis. Moreover, the chitosan produced was evaluated as a flocculant agent for microalgae biomass production. The liquid protein hydrolysate recovered after autolysis showed a protein content of 73.66% (DM basis), representing a good source of animal protein for feed production. The chitin content in 1 kg of shrimp by-product after extraction was 22 g, while produced chitosan obtained was 14 g. Chitosan infrared spectra showed glucosamine bands as described by other works and the degree of deacetylation was calculated as 80,27%. Carotenoids extract showed absorbance profile similar to other shrimp extract, and accounted as total carotenoids for 5.7g/kg of shrimp by-product. The flocculant efficacy of chitosan was comparable to NaOH and aluminum polychloride and superior at a concentration of 5 ppm. These data suggest that biomolecules from *M. rosenbergii* processing by-products are easily recovered using simple steps and have potential for technological applications in biological systems.

Key-words: Protein hydrolysate; chitosan; carotenoids; microalgae flocculants.

7.1 Introduction

Aquaculture is the practice of cultivating aquatic organisms, and it has grown considerably over the years. This growth includes the commercial cultivation of various species of shrimps and prawns intended for human consumption (Ponce-Palafox et al., 2006). When it comes to freshwater shrimps, the "Giant Shrimp" (*Macrobrachium rosenbergii*) is usually considered one of the most important aquaculture species in the world (Pillai et al., 2012). This economic importance is due in large part to their biological characteristics: tolerance to many different environments, rapid growth, relatively large size, good quality of meat and omnivore feeding behavior (Muralisankar et al., 2014).

In aquaculture, the most expensive factor throughout the whole production is the feed costs. Animal protein represent the major bottleneck for feed production, which depends of the organism being farmed and other factors like protein conversion and nutritional quality. The utilization of aquaculture processing waste for nutrients recovery allows the chain of production to be more sustainable, and represents an important source of valuable biomolecules for feed production and other technological applications.

These shrimps, like other crustaceans and insects too, have their exoskeleton composed of chitin, the second most abundant polysaccharide in nature, importante because it is animal origin and also has a fibrous structure (Dutta et al., 2015). This polysaccharide can be modified through deacetylation yielding a compound known as chitosan (Chen et al., 1998).

Chitin and chitosan natural polymers without toxicity, they are also biodegradable and biocompatible in nature. These biomaterials can be easily processed into various forms such as membranes, gels, microparticles, nanoparticles and nanofibers (Anitha et al., 2014).

From the characteristics described these biopolymers have various applications: flocculating microalgae (Gualtieri et al, 1988; Xu et al, 2013; Farid et al, 2013) treatment of industrial pollutants (Songkroah et al., 2004), antimicrobial activity (Salaberria et al. 2014, Dutta et al., 2015; Younes & Rinaudo 2015), production of

nanoparticles with various features (Mincea et al., 2012; Zeng et al., 2012), biomedical and pharmaceutical applications (Yusof et al., 2003; Anitha et al., 2014), among others.

In addition, apart from chitin, other molecules can be extracted from the shrimp processing waste industry: proteins, enzymes, calcium, carotenoids, vitamins and glycosaminoglycans (Cahú et al., 2012). Carotenoids are present as natural pigments, liposoluble, synthetized bybacteria, algae and angiosperms, and animals acquire through the diet.. Today, at least 750 different carotenoids have been identified, these pigments are responsible for the yellow to red color in plants and animals, as well as the variety of stains brown, blue, purple, green and black present in fish and crustaceans (Britton, Liaaen-Jensen; Pfander, 2012).

Thus, the present study aimed in the extraction and characterization of commercially important biomolecules, such as chitin, chitosan, glycosaminoglycans and carotenoids waste processing giant shrimp *Macrobrachium rosenbergii*, in addition to obtaining a protein hydrolysate.

7.2. Material and Methods

7.2.1. Processing waste Raw-material

Fresh heads of shrimp *M. rosenbergii* were obtained from a producer in Recife, PE. The material was collected and placed on ice for transport to the laboratory, then it was weighed and separated into 1 kg plastic bags for freezing at -20°C for later use.

7.2.2. Protein hydrolysate, centesimal composition and fatty acids

The protein hydrolysate was prepared according to Cahu, et al. (2012). The heads were ground with distilled water (at concentration 1: 1), the liquid obtained was placed in a container in a water bath at 45 ± 2°C with constant stirring of 300 rpm for 120 minutes to occur protein hydrolysis. After 120 minutes hydrolysis the material was heated at 100 ° C for 10 minutes to stop hydrolysis. The liquid was then filtered on a 1 mm sieve and the liquid obtained was frozen at -20°C.

The analysis of the chemical composition were made in CBO Analysis Laboratory. The following analyzes were performed: humidity (AOAC, 1996), crude protein (AOAC, 1995a), ether extract (UK, 1982), ash (AOAC, 1995b), carbohydrates, fiber (White et al, 1986; Hagen et al., 1989) and fatty acids (AOAC, 2007)

7.2.3. Extraction of chitin and chitosan preparation

The solid residue which was retained in the preparation of protein hydrolysate was washed with water and dried at 70 ° C. After dry, the residue was treated with 1M HCl for removal of $NaHCO_3$ and 1 M NaOH to deproteinization. For depigmentation it was used potassium permanganate ($KMnO_4$) and 0.5% sodium dithionite ($Na_2S_2O_4$) 1%. The chitin flakes were washed with distilled water and dried at 70°C. The dry chitin was added 500 mL of 50% NaOH in water bath at 65°C for 24 hours with constant stirring. The N-deacetylation procedure was performed twice.

Chitosan was washed with distilled water until reaching neutral pH and then dried at 70 ° C. After drying was pulverized using a mill (IKA ® A11Básico, IKA ® Works, Inc., China) to have particles with 250 micrometers. Then 1% chitosan was solubilised in 3% acetic acid and filtered on filter paper (14 mM), precipitated with 1M NaOH until reaching pH 11 and then the pH was neutralized to pH 7. After the neutralization was purified chitosan centrifuged (10,000 × g for 15 min at 25 ° C) and lyophilized to a fine powder was obtained.

7.2.4. FT-IR spectra of chitin and chitosan

FT-IR spectra of chitin and chitosan samples were measured in KBr pellets in transmission mode within a range of 4000–500 cm^{-1} using an FT-IR Bomem MB100 spectrophotometer. The degree of deacetylation (DD%) was calculated based on the ratio A1320/A1420 reported by Brugnerotto et al. (2001):

$DD\% = 100 - [(A1320/A1420 - 0.3822)/0.03133]$.

7.2.5. Carotenoid pigments extraction

The carapace and the centrifugation precipitate were incubated with 1000 mL 90% (v/v) ethanol each overnight. The ethanol solution was collected and additional ethanol incubation was performed again the ethanolic solution absorbance was performed at 470 nm. The ethanolic solutions obtained from the incubations were collected together (about 2000 mL), concentrated under vacuum at 37ºC using a rotary flash evaporator and dried under N2 atmosphere. From now on, all experiments were undertaken preventing from light conditions under N2 conditions to avoid possible *cis/trans* isomerization and oxidation of the carotenoids and stored at –20ºC until analyses. The approximate amounts of carotenoid amounts in the ethanolic solutions were spectrophotometrically determined (Ultrospec 3000 *pro* spectrophotometer; Bio-Rad) according to the formula (Schiedt and Liaaen-Jensen, 1995): mg carotenoids= (A.vol.1000)/$A^{1\%}_{1cm}$.100 Where A, vol and $A^{1\%}_{1cm}$ stand for the absorbance at 470 nm, volume in mL and the astaxanthin specific absorption coefficient in ethanol, respectively.

7.2.6. Microalgae Culture

The species of microalgae *Chlorella vulgaris* and *Scenedesmus subspicatus* used in this study was obtained from the Culture Centre of Algae of Universidade Federal Rural de Pernambuco, Brazil. The microalgae inoculum were cultivated in semicontinuous culture indoors. The indoor cultures of microalgae were grown with autoclaved freshwater and Provasoli medium in 2 L bottles bubbled with air, incubated in a controlled temperature room (24°C). The bottles were irradiated with daylight fluorescent tubes (light intensity, 4000Lux) for 72 hours. Algal biomass was estimated with use of a Neubauer chamber and optical microscope.

7.2.7 Microalgae Flocculation

The effect of three dosages of a three types of flocculant on microalgal flocculation was studied at room temperature in a 27 1-liter jars for one specie

microalgae. Aluminium polychloride (PAC), NaOH and chitosan at concentrations of 1, 2 and 5 ppm each one were tested. After addition of the flocculating agent was stirred for 30 seconds and the pH was measured using a Hanna HI9828 Multiparameter. After 24 hours of incubation samples were collected and counted the cells with use of a Neubauer chamber.

7.2.8. Data analysis

For data analysis were descriptive statistics used based on percentages through Excel 2007 and statistical inference with normality test (Shapiro-wilk), analysis of variance (ANOVA), Dunnett's test and Tukey's test, with significance level $P < 0.05$, using 7.7 Assistat and GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

7.3. Results

7.3.1. Chemical composition of hydrolysate

The liquid phase is the protein hydrolysate, the analysis of chemical composition are found in table 1. The dry matter content in the present study hydrolyzate was around 4.5% for the hydrolysate *Litopenaeus vannamei* shrimp this content was 9% (Cahú et al., 2012). As the quantity of protein in the dry matter of the hydrolyzate *M. rosenbergii* showed high values even in comparison with hydrolyzate of *O. niloticus* and *L. vannamei* (Table 1) (Robert, et al. 2014; Silva et al. 2014).

Hydrolysates shrimps can be used as ingredients in nutritional supply to other aquatic animals or as a flavoring agents (Cavalheiro, Souza and Bora, 2007; Leal et al. 2010). The use of endogenous enzymes for the hydrolysis of shrimp reduce process costs and have a great capacity in the release of amino acids threonine, serine, valine, isoleucine, tyrosine, histidine and tryptophan (Cahú et al., 2012).

7.3.2. Chitin and Chitosan

After washing the shells pass by the demineralization process, deproteinization and bleached at the end chitin is obtained. Chitin suffers alkaline deacetylation processes to yield chitosan. For a kilogram of shrimp waste was obtained 45g of crude chitin and the end 22g of purified chitin and 14g chitosan (Table 2). Chitosan was further purified and presented complete dissolution in 3% (v/v) acetic acid in water, which demonstrates that chitin was successfully converted to chitosan.

For *Litopennaeus vannamei* were extracted 50g/kg of crude chitin, obtaining a final yield 25g and 17g purified chitin and chitosan, respectively (CAHÚ et al. 2012). In another study were obtained 88g/kg of crude chitin for shrimp species *Penaeus semisulcatus* (Mizani, Aminlari and Khodabandeh, 2005).

To obtain chitin and chitosan from crustacean carapace it is important that the step of deproteinization remove all proteins that are closely associated to the chitin. This process, together with the demineralization will yield a protein concentrate and calcium in amounts that will vary from species to species (Muhlia-Almazán et al. 2002; Coward-Kelly, Agbogbo and Holtzapple, 2006; Cahú et al. 2012).

The FT-IR of chitin and chitosan from *M. rosenbergii* showed in Figure 1. The characteristics found are in accordance with described previously profiles (Paulino et al., 2006).The degree of acetylation for chitosan was calculated to be 80,27%, coffering solubility to chitosan in acidic media. Previous works with chitosan *L. vannamei* showed degrees of deacetylation near 79%, confirming the present results (Cahu et al., 2012). The degree of deacetylation is important because it is he who differs the chitosan from chitin (Kasaai et al., 2000). The bands at 1640–1671 cm^{-1} are attributed to the vibrations of the amide I bond and correspond to the amide I stretching of C O. The band at 1339 cm^{-1} corresponds to a CO-NH deformation of the CH_2 group (amide III) of chitin (Figure 1A). The band at 1556-1560 cm^{-1} corresponds to the stretching or N H deformation of amine II and the bands between 890 and 1160 cm^{-1} represent polysaccharide structures. For chitosan, the band at 1560 cm^{-1} was more intense than that at 1639 cm^{-1}, which suggests effective deacetylation (Figure 1B). When chitin deacetylation occurs, the band observed at 1660 cm^{-1} decreases and then increases again at 1560 cm^{-1}, indicating the presence of NH_2 groups. The band from 1500 to 1700 cm^{-1} demonstrates an intensification of the peak at 1560 and a decrease at 1639-1660 cm^{-1}.˙The band between 3400-3500cm^{-1} correspond to the –OH vibration and is also

characteristic of polysaccharides. The chitin spectrum showed more peaks compared to chitosan, but this is probably due to some extent of contamination with residual proteins in the process of purification.

7.3.3. Carotenoid pigments extraction

The carotenoids extraction scanning spectra are presented in Figure 2. The graphic shows characteristic absorption values for carotenoids in the range between 400 to 500nm. Extracts of *L. vannamei* and *M. rosenbergii* showed a carotenoid amount of 4.08 and 2.85 mg/mL, respectively. *L.vannamei* spectra has a well defined peak for asthaxanthin at 470 nm, that is the major carotenoid component for this shrimp (Cahu et al., 2012; Santos et al., 2012). The extracted carotenoids can be used in the pigmentation of fish meat and feed supplementation (Cano-Lopez et al, 1987; Cavalheiro, Souza and Bora, 2007). The spectra for *M. rosenbergii* presents a broad absorbance range, which suggests that carotenoids molecules are more evenly distributed. Further analyses of the composition of this extract will elucidate the profile and other technological approaches on recovering these pigments from *M. rosenbergii*.

7.3.4. Microalgae flocculation

Cell counts of *Scenedesmus subspicatus* and *Chlorella vulgaris* in flocculation experiments are in Figures 3 and 4, respectively. For *Scenedesmus subspicatus* treatment with concentration of 5 ppm of chitosan was shown that the more efficient flocculation (84,35%). The other chitosan concentrations did not achieve statistical difference with the Sodium hydroxide (NaOH), a flocculating agent previously used, treatments. Aluminum polychloride (PAC) concentrations, showed no flocculation for the specie involved (Fig. 3). All treatments showed pH from 9,58±0,02 to 9,97±0,03 (Table 3).

For Chlorella all flocculating agents showed flocculation with efficiencies between 93.2% and 51.59%. Chitosan 5 ppm was also the most effective (93.2%). For this species the pH varied between 8.04±0,03 and 9.14±0,06 (Table 3).

Several studies show that pH change is very effective for flocculating microalgae, an increase of pH between 8.5 and 10.5 is capable of flocculating microalgae of the species *Phaeodactylum tricornutum* (Sirin et al., 2011), *Anabaena marina* (González-López et al., 2009) or *Dunaliella tertiolecta* (Horiuchi et al., 2003). Chitosan and substances with aluminum in its constitution are widely used in the flocculation of *Chlorella* sp and *P. tricornutum* (Harith et al., 2009; Sirin et al., 2011).

In general the cells of microalgae are negatively charged so the use of cations or cationic polymers are efficient for flocculation of these cells, the Aluminum polychloride and Polyferric sulfate proved efficient (Sirin et al, 2011; Jiang et al. 1993). For *Chlorella* and *Scenedesmus* presents the PAC efficiency below 80% in most cases (Papazi et al., 2010). In the present study the PAC was not efficient in the *Scenedesmus subspicatus* and the *Chlorella* showed maximum efficiency of 77.72% for the concentration of 5 ppm corroborating the studies.

For *Scenedesmus subspicatus* and *Chlorella* chitosan obtained from *M. rosenbergii* to 5 ppm presented flocculation efficiency 84,35% and 93,2%, respectively. Commercial chitosan in a previous state for the same species was not efficient even at high concentrations (Granados et al., 2012). The concentration of 5 ppm is well below the concentrations used for Lubian (1989) (10 ppm to 160 ppm) to flocculate species of marine microalgae with an average efficiency of 75%.

In other study there was a 90% reduction of the turbidity using a commercial chitosan at pH 7 to pH 9 there was no reduction in turbidity (Divakaran and Pillai, 2002). For *Phaeodactylum tricornutum* to commercial chitosan shows efficiency of 90% at 20 ppm and pH 9.9 (Sirin et al., 2012). Wu et al. (2015) demonstrated that 5 ppm of chitosan flocculation efficiency *Scenedesmus sp*. was around 20% reaching its maximum of 90% with a concentration of 20 ppm. In the same study NaOH concentrations reached 2000 ppm 90% efficiency of flocculation.

In most studies NaOH is used for pH flocculation or as an alkali in combination with a flocculating agent to adjust the pH (Harith et al., 2009; Knuckey et al., 2006; Vandamme et al., 2010; Wu et al., 2012; Wu et al., 2015).

The results of the experiments showed that chitosan obtained from *M. rosenbergii*, at a low concentration, was more effective than commercial chitosan and other flocculating agents. This result is very important since this Chitosan is a non-toxic

polymer and was obtained from shrimp waste. Biomass of microalgae recovered with chitosan that can be used in food and aquatic animals because their residues do not offer health risk.

7.4. Conclusions

Shrimp processing wastes are valuable sources of biomolecules, which can be recovered with simple steps. The application of the recovered molecules allows the aquacultured shrimp industry to be more economically viable and environment friendly. Protein hydrolysate with high quality can be used as a substitute for fish meal in feeds; carotenoid pigments are powerful antioxidants and also a required component for fillet coloration of salmonid fish; chitin and chitosan are vastly versatile polysaccharides with many biotechnological applications, such as flocculant agents, but also as antimicrobial, antioxidant and biocompatible fiber. Other biomolecules can be extracted with the present method, such as shrimp oil, glycosaminoglycans and minerals from chitin treatment. More studies are being carried out in order to fully characterize these biomolecules and suggest novel applications.

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Table 1: Chemical composition of the *M. rosenbergii* hydrolysate (%) and others species of aquatic animals.

Nutrient	***Macrobrachium rosenbergii***		***Oreochromis niloticus*** [a]	***Litopenaeus vannamei*** [b]
	Hydrolysate	**DM basis**		
Water content	95,52	0,00	-	-
Protein	3,30	73,66	58,48*	64,50*
Ether Extract	0,61	13,61	37,47*	11,20*
Fiber	0,10	2,20	18,70*	-
Minerals	0,47	10,49	2,67*	11,10*

[a] Silva et al. (2014)

[b] Robert et al. (2014)

*DM basis

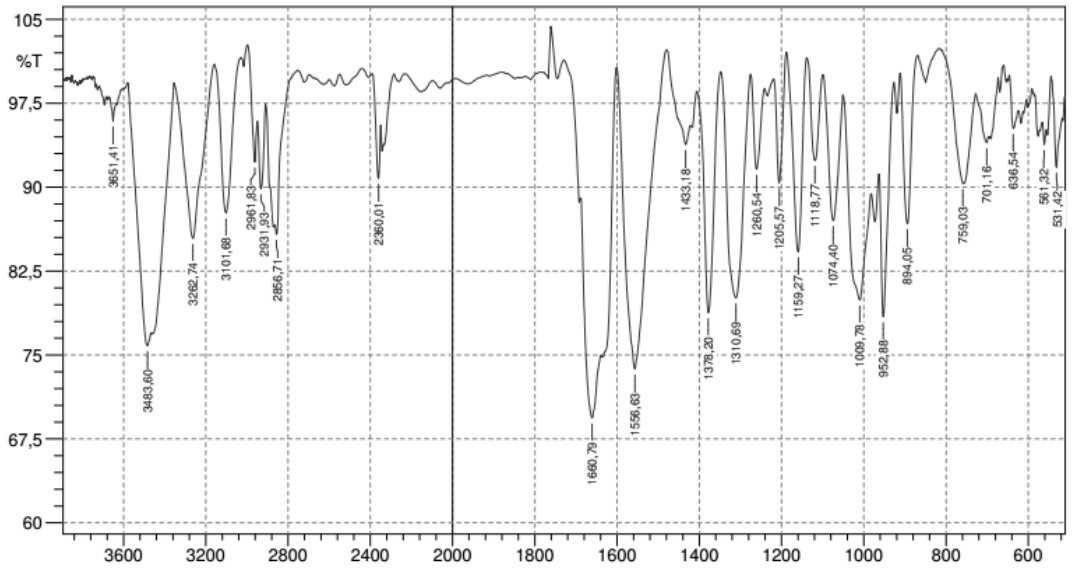
(-) Data not provide by the authors.

Table 2: Chitin and chitosan composition obtained from the shells of shrimps waste *Macrobrachium rosenbergii* and *Litopenaeus vannamei*.

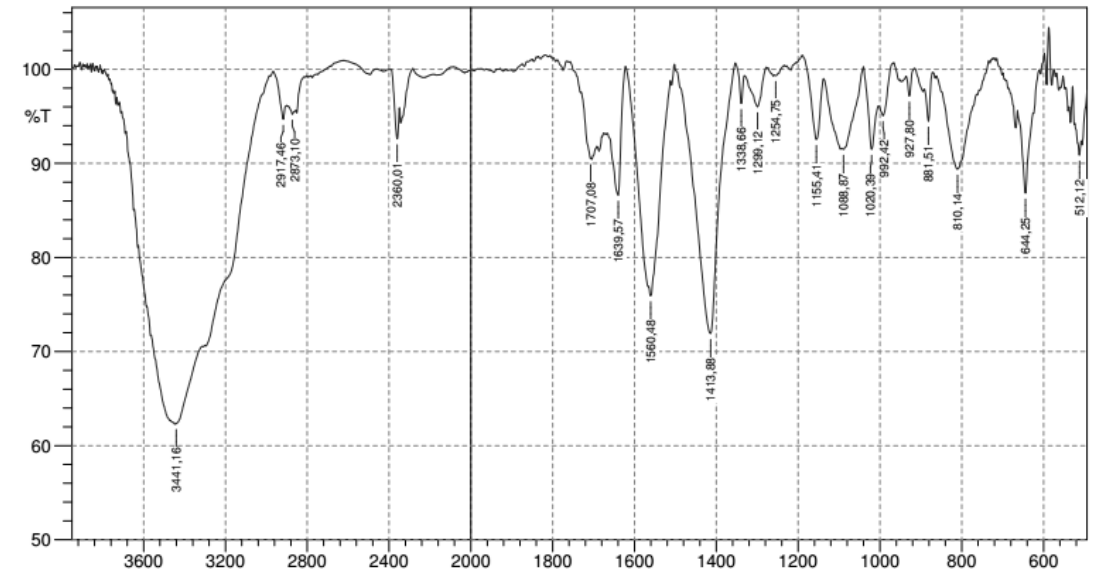
Component	***Macrobrachium rosenbergii***	***Litopenaeus vannamei*** [a]
Chitin	22,00	25,00
Chitosan	14,00	17,00

[a] Cahú et al (2012)

Figure 1: FT-IR chitin (A) and chitosan (B) from *M. rosenbergii*.



A



B

Figure 2: Spectrum of ethanol extracts of carotenoids *L. vannamei* and *M. rosenbergii*.

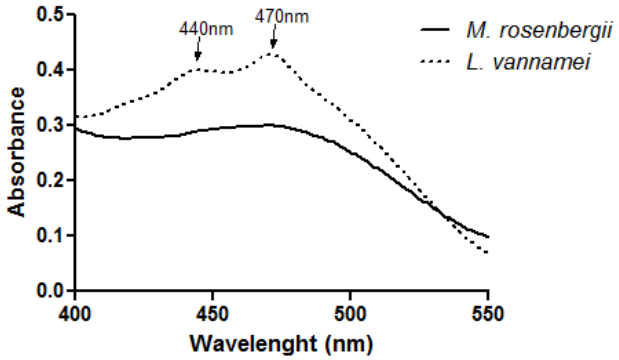
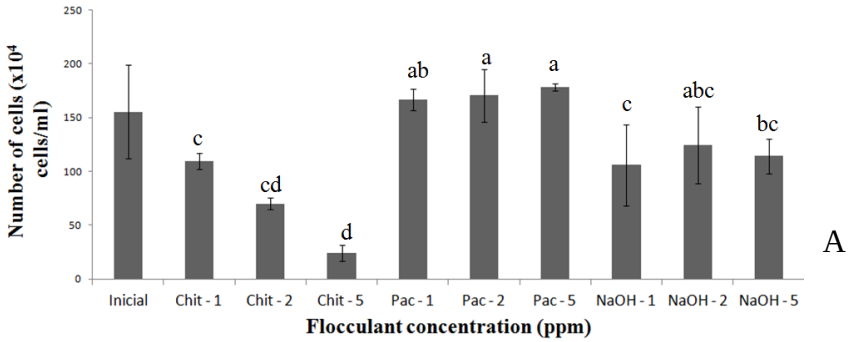


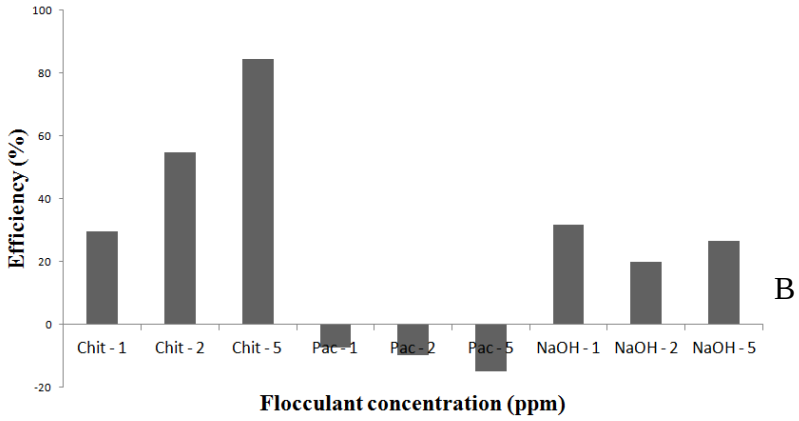
Table 3: After 24h, pH treatments of microalgae species *Scenedesmus subspicatus* and *Chlorella vulgaris*, was exposed to different concentrations of flocculating agents PAC, NaOH and chitosan.

Flocculant agent (ppm)	***Scenedesmus subspicatus***	***Chlorella vulgaris***
Inicial	8,86	8,29
Chitosan 1	9,92±0,06	8,73±0,02
Chitosan 2	9,93±0,02	8,71±0,02
Chitosan 5	9,97±0,03	8,57±0,08
PAC 1	9,69±0,07	9,14±0,06
PAC 2	9,89±0,05	9,00±0,04
PAC 5	9,96±0,01	8,86±0,08
NaOH 1	9,93±0,06	8,23±0,07
NaOH 2	9,58±0,01	8,04±0,03
NaOH 5	9,58±0,02	8,04±0,03

Figure 3: Cell count of *Scenedesmus subspicatus* 24 hours after the addition of flocculant (A) and Efficiency of flocculation (B).



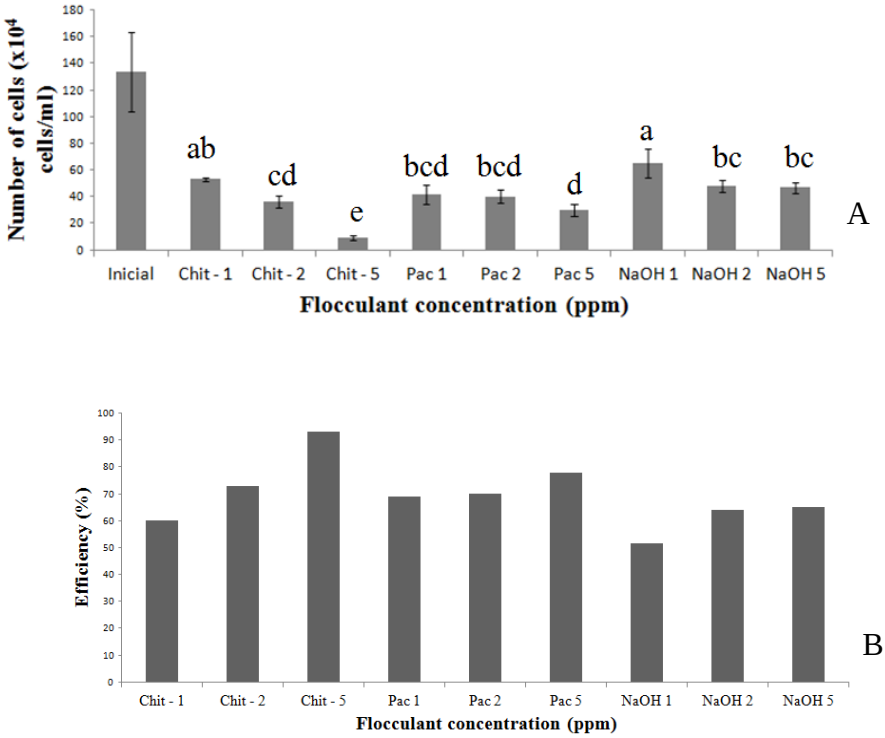
A



B

Different letters represent significant difference.

Figure 4: Cell count of *Chlorella* 24 hours after the addition of flocculant (A) and Efficiency of flocculation (B).



Different letters represent significant difference.

8. Considerações Finais

Todos os anos, toneladas de resíduos do processamento de camarões são levadas a aterros sanitários. O descarte inadequado desse material não só representa mais poluição para aterros e cursos d'água, dado a alta reatividade, como também um ônus para o setor produtor que precisa arcar com os custos do descarte. Esses resíduos são fonte de macromoléculas bioativas que possuem inúmeras aplicações biotecnológicas. O presente trabalho elaborou quatro bioprodutos proteicos como fontes alternativas de proteínas para dietas de peixes, utilizando cabeças de camarão cinza, além de formular uma metodologia para a recuperação de quitina, quitosana e carotenóides dos resíduos do processamento de *M. rosenbergii* cultivado localmente. A recuperação destes biomaterias vem atender à demanda de proteína animal necessária para fabricação de rações e ao crescente mercado por produtos naturais versáteis com função na saúde humana e animal. Seu potencial uso, por exemplo, na aquicultura e na biotecnologia são alternativas promissoras para os produtos convencionais. Constitui-se, assim, uma oportunidade sustentável e de expressão econômica de reaproveitamento dos resíduos.

O agronegócio no Brasil necessita cada vez mais de novas tecnologias para melhoramento de produção, enquanto mantém o sistema viável economicamente e ecologicamente. Os processos biotecnológicos aqui descritos poderão ser aplicados para grandes empresas como também para pequenos produtores, como é o caso dos aquicultores de *M. rosenbergii*. Um bioproduto a base de proteína vegetal e de camarão é passível de ser implementado em rações para cultivo de animais aquáticos, e os resíduos do processamento deste cultivo quando recuperados serão aplicados aos subsequentes, gerando também co-produtos para outras formas de aplicação. Esta abordagem cíclica é uma nova tendência que começa a se estabelecer como protótipo e com potencial para grandes e pequenos produtores.

Anexos

Anexo 1

Guia para submissão de trabalhos ao periódico: Animal Feed Science and Technology

Guide for Authors
1. Original Research Papers (Regular Papers)
2. Review Articles
3. Short Communications
4. Book Reviews

Original Research Papers should report the results of original research. The material should not have been previously published elsewhere, except in a preliminary form.

Review Articles should cover subjects falling within the scope of the journal which are of active current interest.

A *Short Communication* is a concise but complete description of a limited investigation, which will not be included in a later paper. Short Communications should be as completely documented, both by reference to the literature and description of the experimental procedures employed, as a regular paper. They should not occupy more than six printed pages (about 12 manuscript pages, including figures, tables and references).

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Professor G. Flachowsky
Federal Research Centre of Agriculture
Institute of Animal Nutrition
Bundesallee 50
D-38116 Braunschweig
Germany

Manuscripts describing the use of commercial feed products are welcome, but should include the following information: major components, contents of active ingredients (for example enzyme activities). Independent verification, as opposed to a manufacturers guarantee, is always desirable and often avoids difficulties in the review process, especially where there are no, or few, treatment impacts. The Editors reserve the right to reject any manuscript employing such products, wherein this information is not disclosed.

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Submissions concerning NIRS may be suitable when more accurate, precise or robust equations are presented. Mathematical, technical and statistical advancement, may constitute the foundation for acceptance. For more details see the editorial in Vol. 118/3-4.

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Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

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The following definitions should be used, as appropriate:

a. aNDFom-NDF assayed with a heat stable amylase and expressed exclusive of residual ash.
b. NDFom-NDF not assayed with a heat stable amylase and expressed exclusive of residual ash.
c. aNDF-NDF assayed with a heat stable amylase and expressed inclusive of residual ash.
d. NDF-NDF assayed without a heat stable amylase and expressed inclusive of residual ash.
e. ADFom-ADF expressed exclusive of residual ash.
f. ADF-ADF expressed inclusive of residual ash.
g. Lignin (sa)-Lignin determined by solubilization of cellulose with sulphuric acid.
h. Lignin (pm)-Lignin determined by oxidation of lignin with permanganate.

While expressions of NDF and ADF inclusive of residual ash will continue to be acceptable (i.e., the terms aNDF, NDF and ADF above), the Editors-in-Chief highly recommend reporting all fibre values, including digestibilities, on an OM basis. Silica is partially soluble in ND, is quantitatively recovered in AD, and so may contribute to the 'fibre' values and to subsequent digestibility coefficients.

Reporting 'hemicellulose' values as the difference between NDF and ADF is generally only acceptable if the analyses have been sequential on the same sample. Crude fibre (CF), nitrogen-free extract (NFE) and total digestible nutrients (TDN) are not acceptable terms for describing feeds and should only be referred to in a historical context.

Results

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Discussion

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Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

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Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

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Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

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Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

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Anexo 2

Guia para submissão de trabalhos ao periódico: LWT- Food Science and Technology.

INTRODUCTION

LWT - Food Science and Technology is an official journal of the Swiss Society of Food Science and Technology (SGLWT/SOSSTA) and the International Union of Food Science and Technology (IUFoST). *LWT - Food Science and Technology* is an international journal that publishes innovative papers in the fields of food chemistry, biochemistry, microbiology, technology and nutrition. The work described should be innovative either in the approach or in the methods used. The significance of the results either for the science community or for the food industry must also be specified. Contributions that do not fulfil these requirements will not be considered for review and publication. Submission of a paper will be held to imply that it presents original research, that it has not been published previously, and that it is not under consideration for publication elsewhere.

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e. Total number of figures ≤ 5.

f. Maximum number of references (including those cited in tables and figures) not to exceed 50.

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Anexo 3

Guia para submissão de trabalhos ao periódico: International Journal of Biological Macromolecules.

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