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BIOTECHNOLOGY

Bacteriocin-like inhibitory substance in aquaculture: a classic method of protein precipitation for a new aplicability

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ABSTRACT. Techniques to decrease losses from bacterial diseases are always important to improve the fish production. The use of antagonistic substances (bacteriocins) has been proven to be a viable option. The aim of this study was to evaluate different methods of purification for bacteriocin like inhibitory substances (BLIS). For the purification process, we isolated and used two Gram-positive bacilli that produce antagonistic substances for pathogens in aquaculture. Tests for detection of interfering factors were also performed. After the confirmation that the antagonistic action was due the BLIS activity, we carried out the purification methods. The methods tested were: cell free supernatant, acid extraction and ammonium sulfate precipitation at two concentrations (20 and 50%). *Salmonella* Tiphy CFP/IAL1472 and *Aeromonas hydrophila* (isolated in a tilapia production environment) were used as indicators of the efficiency of extracts in controlling pathogenic potentials. Ammonium sulfate precipitation at 50% was the most appropriate for purifying the antagonistic substance for both indicators. The extracts of the two isolates remained active for 22 days at 25ºC. These are promising results regarding the water and fish health without the use of antibiotics, in this manner being a safer environmental practice.

Keywords: bacteriocins; Gram-positive bacilli; tilapia culture; bacteriocin-like inhibitory substance purification.

Substância inibidora tipo bacteriocina na aquicultura: método clássico de precipitação de proteínas para novas aplicações

RESUMO. Técnicas para diminuir as perdas causadas por doenças bacterianas são importantes para melhorar continuamente a produção de pescado. O uso de substâncias antagônicas (bacteriocinas) tem-se mostrado uma opção viável. O objetivo do trabalho foi avaliar diferentes métodos de purificação de bacteriocinas como substâncias inibidoras (BLIS). Dois bacilos Gram-positivos, produtores de substâncias antagonistas para agentes patogênicos da aquicultura, foram utilizados em processos de purificação. Depois de confirmada a ação antagônica pela atividade de BLIS, os métodos de purificação foram realizados. Os métodos testados foram: células livres de sobrenadante, extração ácida e precipitação por sulfato de amônia em duas concentrações (20 e 50%). *Salmonella* Typhi PCP/IAL1472 e *Aeromonas hydrophila* (isolada de um ambiente de tilapicultura) foram utilizadas como indicadores de eficiência dos extratos. O precipitado por sulfato de amônio a 50% foi o mais adequado para purificar a substância antagonista para ambos os isolados indicadores. Os extratos dos dois isolados permaneceram ativos por 22 dias em 25ºC. Estes resultados são promissores do ponto de vista da manutenção da sanidade da água e dos peixes, sem uso de antibióticos, constituindo uma prática ambientalmente mais segura.

Palavras-chave: bacteriocinas; bacilos Gram-positivos; tilapicultura; purificação de substâncias antagonistas tipo bacteriocina.

Introduction

Bacterial diseases are usually a problem in aquaculture. *Aeromonas hydrophila* and *Pseudomonas* sp. are common fish pathogens for Tilapia, but other microorganisms, including human pathogens, can also be the cause of problems (Longyant, Chaiyasittrakul, Rukpratanporn, Chaivisuthangkura, & Sitthigorngul, 2010; Zhang, Zhou, An, Chen, & Wang, 2014). The diseases caused by pathogens lead to losses in production or even delayed growth in the fish. Thus, handling practices that reduce the impact of bacterial disorders on fish production are necessary. Many illnesses of bacterial origin could be avoided, including the emerging diseases caused by *Francisella* sp. (Soto, Hawke, Fernandez, & Morales, 2009), *Lactococcus garviae* (Evans, Klesius, & Shoemaker, 2009) and bacteria from the Midichloriaceae family, from the Rickettsiales Order (Cafiso et al., 2015).

The bacterial contamination is also important on the post fishing, because it can influence in the shelf life and fish consumption. Species from the *Salmonella* genus are strongly linked to contamination in the handling process. The bacterial contamination may reduce the shelf life and is strongly involved in human food poisoning (Bartolomeu, Dallabona, Macedo, & Kirschnik, 2011; Gatti Junior, Assunção, Baldin, & Amaral, 2014). The *Salmonella* spp., including *Salmonella* Typhi, have been isolated in more than 30% of the fish from the Lake Victoria, in Kenya (David, Wandilli, Kakai, & Waindi, 2009).

Antibiotics are normally used to minimize the damage caused by pathogenic microorganisms, both in therapeutic and in prophylactic applications. They present low toxicity to fish and are largely stable during a specific time. However, these compounds can be carried into other environments, which create selective pressures and trigger alterations in the environmental microbiota (Resende et al., 2012). The resistant bacteria selected by the antibiotic use can be harmful to fish, to human and even to the environment, which leads to the need to develop new medicine and/or technology to reverse this situation (Burridge, Weiss, Cabello, Pizarro, & Bostick, 2010; Li et al., 2015). It is already known that drug-resistant bacteria occur in the aquatic environment in the aquaculture systems (Resende et al., 2016).

Another way to maintain a healthier aquaculture production is by oral vaccines. However, it is difficult to create vaccines with long-term immune-protective effectiveness, as most of those provoke just a local immune response (Li et al., 2015).

The use of probiotics has gained popularity in aquaculture in recent years, as they improve the immune-modulating capacity of the fish by occupying niches, by its antagonistic substance production and/or by the improvement in their hosts' capacity to absorb nutrients (Verschuere, Rombaut, Sorgeloos, & Verstraete, 2000; Mello et al., 2013). However, the employment of probiotics requires the use of living microorganisms to achieve their effects (Gatesoupe, 1999). Many of the probiotic bacteria produce antagonistic substances like bacteriocins, which are capable to control the growth of disease-causing microorganisms (Vaseeharan & Ramasamy, 2003; Silva et al., 2012).

From this perspective, antagonistic substances, like bacteriocins, are considered safe when they are added to environments of production of aquatic animals. Bacteriocins are peptides with specific action, low toxicity and low environmental impact, as well as being cheap to extract. Thus, the aim of this study was to evaluate three different methods of extracting Bacteriocin-Like Inhibitory Substances (BLIS).

Material and methods

We identified two Gram-Positive bacilli strains (C2I12 and C2I13 – producer strains), previously isolated from Nile tilapia (*Oreochromis niloticus*) intestine (Del'Duca, Cesar, Diniz, & Abreu, 2013), as potential producers of BLIS. These isolates were submitted to different types of protein extraction, in order to test the action of the antagonist substance against potentially pathogenic bacteria species of interest in aquaculture (*Salmonella* Typhi CFP/IAL1472 and *A. hydrophila* [isolated in an environment of tilapia production]).

CI12 and C2I13 identification

To identify the potential producers of BLIS, the 16S sequencing of the bacteria RNA was performed. The sequences obtained were compared with GenBank database using the Basic Local Alignment Search Tool for Nucleotide – BLASTN.6.

Detection of interfering factors

To characterize the antagonistic substance as BLIS, tests for the detection of interfering factors were performed. To exclude the possible presence of bacteriophages, a piece of agar (3 mm in diameter) from the inhibition zone was removed aseptically. Then, the agar blocks were homogenized, centrifuged, placed onto the surface of BHIA and overlaid with 3.5 mL of BHI soft agar (0.7%), which contained the indicator strain. After 24 hours of incubation under aerobiosis, the Petri dishes were evaluated for the presence of lytic zones. To exclude a possible inhibition by long chain fatty acids, we conducted tests on BHIA, with and without the addition of 1% soluble starch. To exclude acidic pH as responsible for the antagonism, the BHIA was prepared supplemented with a pH indicator that allowed the pH change in the medium by means of color change around the BLIS-producing strain. Finally, in order to exclude a possible interference of the residual chloroform, used to detect the antagonism by the double layer diffusion method, the antagonistic activity was also determined without the use of chloroform. After the growth of the producing strains, the indicator strains were inoculated with a loop near the spots of the producer strains without previous exposure to chloroform. The cultures were incubated at 37ºC for 24 hours, under aerobiosis, and then examined for the presence of inhibition zones.

Extract obtainment

a) Acid extract:

The pH of the culture (24 hours at 35°C with the inoculum pre-adjusted to match that of a 0.5 McFarland standard - 1.5 x 10^8 CFU x mL^{-1}) was adjusted to pH 7.0 with 5M NaOH. The cells were collected by centrifugation, they were washed twice with 5 mM sodium phosphate buffer (pH 7.0), resuspended in 0,1M NaCl solution (pH 2.0, adjusted with HCl) and submitted at 4°C for 4 hours to release the bacteriocin molecules from the cell surface. The cells were separated by centrifugation (18000 x g for 20 minutes at 4ºC) and the supernatant was adjusted to pH 5.0 with sterile NaOH.

b) Cell-free culture supernatant:

Cell-free supernatant of each tested strains were obtained from a culture (24 hours, at 35°C with the inoculum pre-adjusted to match that of a 0.5 McFarland standard - 1.5 x 10^8 CFU x mL^{-1}) by vacuum filtration system, with the use of a 0.22 µm pore size membrane.

c) Precipitation with ammonium sulfate with 20% and 50% of saturation:

After obtaining the cell-free supernatant, as described above, $(NH_4)_2SO_4$ was added to achieve 20% of saturation or 50% of saturation in independent samples. When the dissolution was complete, the samples were centrifuged (18000 x g for 20 minutes at 4ºC). The supernatant was discarded and the pellet was re-suspended in sterile distilled water.

Stability tests

After obtaining the extracts, they were stored at different temperatures (-4, 4, 25 and 37ºC) until 30 days, in order to evaluate the stability. At specific times, the extracts were serially diluted twofold with sterilized distilled water to obtain the concentrations 1:1; 1:2; 1:4; 1:8; 1:16; 1:32 (v/v). The activity was then assayed against the indicator strain *A. hydrophila* and *Salmonella* Typhi by the method of agar well diffusion.

We spread 100 µL, pre-ajusted to 0.5 McFarland standard, of the indicator bacterial suspensions cultured for 24 hours on the plates, and wells of 10 mm of diameter were punched in the agar with a sterile tip. Then, 20 μL of each concentration was added into the wells. As the control for the inhibition zones, we used distilled water, and $(NH_4)_2SO_4$ aqueous solution at 20 and 50%. The plates were then incubated at 37°C overnight. The extract that did not undergo dilution was named crude extract.

Results

The C2I13 was identified as *Bacillus* sp. Its identity obtained 99% similarity to the 16S subunit of the bacterial RNA compared in the GenBank. The C2I12 sequencing did not provide reliability in the obtained sequence, therefore, it must be reprocessed.

The experiments for detection of interfering factors confirmed that the antagonistic activity in the indicator' strain growth in the inhibition zone was not related to the residual chloroform, bacteriophage activity, long chain fatty acids production, or acidic pH. Thus, the inhibition zone is a consequence of the bacteriocin production by C2I12 and C2I13.

The tests with the cell-free supernatant, acid extraction and precipitation with ammonium sulphate at 20% for the two producer strains showed negative results, i.e., there were no inhibition zones of growth of *A. hydrophila* and *Salmonella* Typhi identified by the agar well diffusion method.

The BLIS extraction, made by precipitation with ammonium sulphate at 50%, showed a positive result for the two isolates tested for both indicator species, since zones of inhibition of growth of *A. hydrophila* and *Salmonella* Typhi were present.

The crude extract with ammonium sulphate at 50% of both strains showed activity during 22 days, when it was stored at room temperature (around 25°C). The extract of C2I12 showed antagonistic activity for up to 15 days when diluted at 1:2 and when stored at 7°C; The extract of C2I13 showed antagonism when diluted at 1:2 and when stored up to 37ºC (Table 1 and 2).

Table 1. Stability of the extract by ammonium sulfate precipitation at 50% (p/v) for C2I12 under thermic treatment over time. (1:8 was the highest dilution with antagonistic activity).

Temp. / Time	-4ºC	4ºC	25ºC	3ºC	60ºC
15 min.	-	-	-	1:4	1:2
30 min.	-	-	1:4	1:2	-
1 hour	-	1:4	1:2	1:2	-
1.5 hour	-	-	-	1:2	-
2 hours	-	1:2	1:2	1:2	-
24 hours	1:2	1:4	1:2	CRUDE	-
48 hours	1:2	1:4	1:2	CRUDE	-
15 days	1:2	1:2	-	CRUDE	-
22 days	CRUDE	CRUDE	CRUDE	-	-

- Not tested or absence of antagonistic activity.

Table 2. Stability of the extract by ammonium sulfate precipitation at 50 % (p/v) for C2I13 under thermic treatment over time. (1:16 was the highest dilution with antagonistic activity).

Temp. / Time	-4ºC	4ºC	25ºC	37ºC	60ºC
15 min.	-	-	-	1:2	1:2
30 min.	-	-	1:2	1:2	-
1 hour	-	1:8	1:2	1:1	-
1.5 hour	-	-	-	1:1	-
2 hours	-	1:2	1:1	1:1	-
24 hours	1:2	1:2	1:2	1:2	-
48 hours	1:4	1:4	1:4	1:4	-
15 days	1:4	1:4	1:2	1:2	-
22 days	CRUDE	CRUDE	CRUDE	CRUDE	-

- Not tested or absence of antagonistic activity.

Discussion

New strategies to control bacterial infections in aquaculture are important. Several biocontrol measures have been assayed as an alternative to antibiotic use (Defoirdt, Sorgeloos, & Bossier, 2011), and among these alternatives, the use of bacteriocins in aquaculture has been discussed. The study conducted by Vijayabaskar and Somasundaram (2008) and Touraki, Frydas, Karamanlidou, and Mamara (2012) have shown bacteriocin activity from the *Bacillus* spp. species against fish pathogens.

One of the isolates identified in our research was a *Bacillus* sp. The bacteria of this genus have generally been isolated from fish intestines, and they are recognized as producers of bacteriocins with great potential to be used in aquaculture (Dong, Yi, Liang, and Shi, 2017; Yi et al., 2018). The non-reliability in the identification of the other isolate that was tested was generated by a noise in the analysis. Therefore, it is important to reprocess this sample.

The test with the cell-free supernatant from the two tested strains against *Salmonella* Typhi and *A. hydrophila* showed no positive results. Probably, when they were diluted in supernatant, the evaluated BLIS was not able to affect the growth of these two species of indicator bacteria. Thus, is necessary to concentrate and extract the substances to obtain the antagonistic action. Likewise, the extracts of C2I12 and C2I13 obtained by acid extraction and by precipitation with 20% of ammonium sulfate presented negative results. These results may indicate that the antagonistic substance could not be obtained by acid treatment, since the acid causes denaturation of the peptide and, consequently, the loss of their function. The same hypothesis is considered for the precipitation made with the ammonium sulphate up to 20% of saturation.

Therdtatha et al. (2016) also did not find any significant results when conducting the cell-free supernatant test and the 20% ammonium sulphate saturation precipitation as a partial method of salivaricin purification, which was obtained from a *Lactobacillus salivarius* culture, against a variety of species of bacteria, including *A. hydrophila* and *Salmonella enterica* sorovar Enteritidis. Sankarankutty and Palav (2016) worked with the acid extraction method for the partial purification of a bacteriocin from lactic acid producing bacteria. They observed that this method of extraction was not suitable for the bacteriocin in question, possibly because it was an *in natura* peptide. Thus, it can be assumed that the antagonist substances extracted from C2I12 and C2I13 are also *in natura* peptides.

At 50% concentration of salt, the peptides extracted from C2I12 and C2I13 showed antagonistic activity against the indicator samples (*A. hydrophila* and *Salmonella* Typhi), which shows that this percentage of ammonium sulfate is sufficient to partially purify antagonistic substances from the two Gram-positive isolates. This form of extraction was also successfully used by Abdel-Haliem, Tartour, and Enan (2016) for bacteriocins from *Lactobacillus plantarum*, isolated from preserved olives.

The stability and efficiency of both extracts obtained by 50% (p/v) concentration of ammonium sulfate for the control of potential pathogens for up to 22 days is an important result, and since this is a relatively long period, the product can be applied less often. Thus, it indicates that the application of the extract containing the antagonist substance could be done every 20 days, on average, which reduces the costs of production when compared to other products that require daily or weekly application for the sanitary control of water and of aquaculture animals.

It is possible to affirm that the use of this type of substance in aquaculture is promising. This is because the peptides produced by the two isolated Gram-positive bacteria showed activity against important pathogens at the range of temperature several tropical species, in addition to present low environmental risks, which have been some considerable concerns of fish producers.

The temperature of stability and activity of the bacterial extracts, which remains up to 25°C, presents advantages for the application in aquaculture production in Brazil and in other tropical countries, since the average temperatures of those countries are around 25°C.

Conclusion

The best method for extracting the antagonist substances in question was the ammonium sulfate precipitation at 50%, which indicates that the desired peptides, i.e. those with antagonistic activity, are extracted at the saturation rate between 20 and 50% with ammonium sulfate.

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