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Importance of the C/N-ratio on biomass production and antimicrobial activity from marine bacteria *Pseudoalteromonas* sp

Importancia de la relación C/N en la producción de biomasa y actividad antimicrobiana de la bacteria marina *Pseudoalteromonas* sp

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Abstract

The main objective of this study was to investigate from the physiological point of view, the influence that nitrogen and carbon sources have on the biomass production and activity of the antimicrobial protein of *Pseudoalteromonas* sp. The impact of the change in the medium initial C/N-ratio on biomass production and antimicrobial activity against *S. aureus* MRSA was evaluated using two different carbon sources (glucose or citrate) and a fixed concentration of NH_4Cl as a nitrogen source. The change in total nitrogen concentration in the medium was evaluated using organic or inorganic nitrogen sources (NH_4Cl or yeast extract-peptone) while a fixed glucose concentrations was present.

The results showed that the increase in C/N-ratio stimulated biomass production but inhibited antimicrobial activity regardless of the nature of the carbon source. Similarly, the biomass profile and antibiotic activity were dependent in the variation of low nitrogen total concentrations in the medium and especially at concentrations bigger than 3 g L^{-1} negatively affected the antimicrobial activity. This indicated that the main factor affected the secondary metabolism of *Pseudoalteromonas* sp. was the nitrogen concentration over C/N-ratio.

Keywords: C/N-ratio, nitrogen, antimicrobial, marine bacterium, *Pseudoalteromonas*.

Resumen

El principal objetivo de este estudio fue investigar desde el punto de vista fisiológico, la influencia que tienen las fuentes de nitrógeno y carbono en la producción de biomasa y actividad antimicrobiana de *Pseudoalteromonas* sp. Se evaluó el impacto del cambio en la relación C/N inicial del medio de cultivo en la producción de biomasa y actividad antimicrobiana contra *S. aureus* MRSA, utilizando dos fuentes de carbono diferentes (glucosa o citrato) y como fuente de nitrógeno, una concentración fija de NH_4Cl. Se evaluó el cambio de la concentración de nitrógeno total en el medio utilizando fuentes de nitrógeno inorgánica u orgánica (NH_4Cl o extracto de levadura-peptona) mientras estuvo presente una concentración fija de glucosa.

Los resultados mostraron que el incremento en la relación C/N estimuló la producción de biomasa pero inhibió la actividad antimicrobiana sin importar la naturaleza de la fuente de carbono. Similarmente, el perfil de biomasa y actividad antibiótica fueron dependientes en la variación de bajas concentraciones de nitrógeno total del medio y especialmente a concentraciones mayores de 3 g L^{-1} afectaron negativamente la actividad antimicrobiana. Esto indicó que el principal factor que afectó el metabolismo secundario de *Pseudoalteromonas* sp. fue la concentración de nitrógeno sobre la relación C/N.

Palabras clave: Relación C/N, nitrógeno, antimicrobiano, bacteria marina, *Pseudoalteromonas*.

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1 Introduction

Marine ecosystems are the largest on Earth. They are made up of a great variety of life forms that interact with each other and are subject to extreme variations of abiotic factors such as salinity, temperature, pressure, light, chemical composition and substrates, among others.

In order to face these variations, marine microorganisms have exclusive metabolic and physiological machineries that produce a great variety of compounds with unique chemical structures not observed in terrestrial microorganisms (McCauley *et al*., 2020). Some compounds are produced in response to stress and they could have biotechnological or pharmaceutical applications due to the growing demand for new drugs, such as antimicrobials (Kazmaier and Junk, 2021; Masschelein *et al*., 2017; Papon *et al*., 2022).

It is currently known that the production of antimicrobial substances by terrestrial microorganisms -such as actinobacteria- is mainly regulated by the sources of carbon, nitrogen and phosphorus (Kong *et al*., 2019). However, in the case of marine bacteria, little is known about the metabolic and physiological processes involved in the production of bioactive metabolites or if the regulatory mechanisms are similar to those found in terrestrial bacteria. Understanding these regulatory processes involved in the biosynthesis of antibiotics and their interaction with the microorganism that produces them is important to increase the availability of the metabolite which could be interesting for biotechnological research.

Pseudoalteromonas is a genus of marine bacteria whose biotechnological importance lies in the fact that many of its species produce various molecules as an adaptive response to demanding or competitive living conditions, so some of them can inhibit the growth of other organisms (Offret *et al*., 2016).

Previously, it was determined that the antimicrobial activity of *Psedoalteromonas* sp. was exerted by a protein that has proved to be effective against the infectious agent *S. aureus* MRSA -which is an agent of emerging diseases-. This secondary metabolite showed similar minimum inhibitory concentration of 1 μg mL^{-1} than vancomycin on *in vitro* study (Cetina *et al*, 2010). However, the quantity and biological activity of this protein produced by *Pseudoalteromonas* sp. are determined by the variation of environmental conditions which affect the growth rate and secondary metabolism (Commichau *et al*., 2006; Pham *et al*., 2019). For example: in the decomposition process of organic matter, the potential for microbial growth can be determined by the actual availability of nitrogen and carbon and by the carbon:nitrogen ratio (C/N) rather than the trophic level, that is, the low C/N-ratio (1.5 - 3.5) in the medium favored the decomposition speed compared to a high C/N-ratio (Hassan *et al*., 2020). While variations of the C/N-ratio (37, 74 and 111) in the culture medium of terrestrial bacterium *Rhyzobium phaseoli* cultures positively impacted the bacterial growth observing higher biomass production at C/N-ratio 74 when bacterium grown on glucose. Conversely, when fructose was used only at low C/N-ratio of 37, the growth was observed (García-Cabrera *et al*., 2021).

In this order of ideas, environments with easily metabolizable nitrogen sources where high concentration of nitrogen (100 g/L) and limitation of the carbon source are detected, they favored the growth of marine microorganisms such as *Arthrinium c.f. saccharicola*, resulted in a negative impact on secondary metabolism while the low concentration of nitrogen source favored its stimulation (Miao *et al*., 2006).

In the marine ecosystem, concentration of C and N is complex and heterotrophic bacteria can use preformed organic compounds (amino acids, purines and pyrimidines) as source of nitrogen and carbon simultaneously with NH_4^+ or NO_3^- (Berman and Bronk, 2003, Damashek and Francis, 2018). Therefore, the production of a secondary metabolite also depends on the type and concentration of the nitrogen source. The experiments of Kroer *et al*. (1994) with marine bacteria showed that in addition to a low C/N-ratio of 5, the concentrations of complex organic amino acids in the sea were more important for the growth of the bacteria, as long as other inorganic and organic compounds were available. However, the basic knowledge of the physiological and biochemical factors that production control and biological activity of secondary metabolites is still very limited. This lack of information compromises the interpretation of the effect that C/N-ratio has in the marine microorganisms.

Therefore, to explain the causes that mediate the relationship between nutrient availability and the type and quantity of secondary metabolites produced in marine bacteria, it is suggested that high C/N-ratio (> 8) could stimulate the production and/or the antimicrobial activity than a low C/N-ratio without carbon limitation which would favor the development of marine bacteria growing on complex substrates such as peptone. Thus, our main objective was to study -from the physiological point of view- the influence that the nitrogen and carbon source have on the biomass production and antimicrobial activity of *Pseudoalteromonas* sp. by understanding the relationships between the type and nutrient availability versus biological activity of secondary metabolites produced while analyzing the C/N-ratio.

2 Materials and methods

2.1 Material

Ammonium chloride, sodium citrate, D-glucose, iron citrate, sodium chloride, magnesium chloride hexahydrate, magnesium sulphate, calcium chloride dihydrate, potassium chloride and sodium bicarbonate were purchased from Sigma-Aldrich (Campeche, Mexico). Peptone and yeast extract were purchased from Bioxon (Campeche, Mexico).

2.2 Microorganisms

The marine bacterium used in this study was obtained from sediment samples in the Gulf of Mexico; Campeche as previously described (Cetina *et al*. 2010). The strain was identified based on its molecular 16S rRNA gene sequence as *Pseudoalteromonas* sp. In addition, the antimicrobial activity of its biomass against human pathogens was reported as secondary metabolite. The human pathogenic *Staphylococcus aureus* (MRSA) was grown in CST broth at 37 °C for 24 h and used for evaluating the antibacterial activity.

2.3 Media and cultivation conditions

A pre-culture was prepared by inoculating 0.1 mL of *Pseudoalteromonas* sp. from our frozen stock to 25 mL of growth medium in a 125 mL flask and left to grow for 24 h at 28 °C on an orbital shaker at 150 rev. min^{-1}. The YPG (yeast extract-peptone-glucose) growth medium was prepared as follows (g L^{-1}): yeast extract, 4.0; peptone, 5.0; glucose, 10; iron citrate, 0.001. All components were dissolved in filtered natural seawater and adjusted to pH 7.4.

2.4 Effect of C/N-ratio with glucose or sodium citrate as carbon sources

In order to elucidate the response of *Pseudoalteromonas* sp. to different C/N-ratio and nitrogen concentration in the growth medium, batch culture experiments were carried out, changing one component at a time and the others remaining constant. The growth medium was prepared with a constant initial concentration of ammonium chloride and variable concentrations of glucose to generate different C/N-ratio (2.75, 5.5, 11, 22 or 55). In the same way, the effect of the C/N-ratio was evaluated with different concentrations of sodium citrate (a rapidly assimilable carbon source) maintaining the concentration of ammonium chloride constant to generate low C/N-ratio (0.05, 0.11, 0.22, 0.55 or 1.1). The pH was adjusted and controlled at 7.4 during the whole protocol.

2.5 Effect of total nitrogen concentration with organic or inorganic nitrogen sources

To elucidate the impact of the initial total nitrogen content of the medium on the biomass production and antimicrobial activity of *Pseudoalteromonas* sp, the medium was prepared with a constant C/N-ratio of 2.75: 1 with glucose as a carbon source and different concentrations of ammonium chloride (1.4, 3.64, 4.55, 9.1, 18.2, 36.41, 91.02 mM) as an inorganic nitrogen source. On the other hand, to evaluate the effect of nitrogen concentration with an organic source, a rich complex media was prepared with different concentrations of yeast extract-peptone to obtain total nitrogen concentrations of 1.65, 3.29, 3.71, 7.39, 8.29, 11.12, 14.77, 16.45, 24.72, 29.62, 32.91, 74.15 mM.

2.6 Effect of the osmolarity of the media

With the data of the solute composition of the culture media, the osmolarity values were calculated (0.116, 0.233, 0.466, 0.923 and 2.329 Osmol L^{-1}).

2.7 Measurement of carbon and organic nitrogen concentration in the substrates

Total organic carbon concentration of yeast extract and peptone was determined by a semi-automatic organic carbon analyzer, Total Organic Carbon (Shimadzu TOC-Vcsn). The total nitrogen concentrations in substrates were determined by the Kjehdahl nitrogen method.

2.8 *Determination of biomass as cell dry weight*

A 25 mL amount of cultured marine bacteria was filtered through GSWP 0.22 μm-pore-size Millipore membranes under vacuum; the mass retained on the filter was washed with 25 mL of sterile natural seawater. The cells were dried in a microwave oven (780 W) with carousel set to 60 % of the maximum power in successive cycles of 1 min until constant weight. Constant weight was considered when the value does not change after three consecutive weighing. Every determination from each treatment was performed thrice and the corresponding averages were reported.

2.9 *Crude extract*

For the antimicrobial activity assay, a crude ethanolic extract was prepared from the microbial biomass of *Pseudoalteromonas* sp. free of culture medium as previously described by Lopez *et al*. (2012). Marine bacteria cells were separated from the spent broth by centrifugation (at 3000 G for 15 min at 4°C) and washed with sterile natural seawater twice. Two grams of biomass were suspended in 10 mL of PBS and sonicated. Nine volumes of cold ethanol 100% were subsequently added to one volume of sonicated solution, mixed and kept for at least 10 min at -20 °C. The mix was centrifuged for 15 min at 4 °C at 15000 G. The pellet retained was washed with cold 90% ethanol and dried (by inversion on tissue paper). The dried sample was suspended in 50 mM PBS, pH 7.0, to adjust protein concentration to 4.0 mg mL^{-1}. This crude extract was used to evaluate antimicrobial activity against pathogen bacteria using the conventional diffusion plate method.

2.10 *Antimicrobial activity assay*

The antibacterial activity of the crude extract was tested by the standard disc diffusion (Kirby-Bauer) method against the Gram-positive bacterium *Staphylococcus aureus* MRSA as reference microorganism. Paper discs (6 mm) were saturated with 20 μL of crude extract. The paper discs were placed onto the surface of Muller Hinton agar containing the test microorganisms. Clear zones of inhibition around the discs indicated antibacterial activity, which was measured after 24 h of incubation at 37°C. Vancomycin and PBS, 50 mM were used on the plates as the positive or negative control, respectively.

The specific activity of antimicrobial substance was standardized according to the National Committee for Clinical Laboratory Standards (NCCLS, 1993). One unit of antimicrobial activity was defined as the quantity of the active substance giving rise to the same level of inhibition as 1 μg of vancomycin. As a general rule, an inhibition diameter less than 11 mm was indicative of a resistant strain.

2.11 *Statistical analysis*

Given values are means ± SD of three replicates per cell line and treatment. ANOVA test using Holm-Sidak algoritm was used to determine significance at $p < 0.005$ level. All statistics were conducted using the SigmaStat v. 12 software (Systat Software Inc., San Jose, CA, USA).

3 Results and discussion

3.1 *Effect of variation of the C/N-ratio in a medium containing glucose or citrate as carbon sources*

Environmental conditions that affect growth and secondary metabolism of microorganisms are closely related to the composition and concentration of nutrients. To face the variation of these physical and chemical factors, microorganisms adapt their metabolism through the regulation of their biochemical pathways that determine the quantity, structural diversity and biological activity of the metabolites produced (Commichau *et al*., 2006; Pan *et al*., 2019; Hernandez-Rosas *et al*., 2021). The marine flora and fauna of the Gulf of Campeche copes with variations in environmental parameters typical of the region, including the distribution of total organic carbon (TOC) and total nitrogen (TN) where the average value of the C/N ratio is 8.0 +/ - 1.7 according to Escobar-Briones and Garcia-Villalobos (2009). For many marine bacteria, the assimilation of glucose as a carbon source is common and constitutes more than 15 % of the dissolved organic matter (DOM) in this ecosystem (Klingner *et al*., 2015). In our previous work (López *et al*., 2012), *Pseudoalteromonas* sp. was grown in a medium containing glucose as a carbon source to produce in the ideophase an antimicrobial protein of 80 kDa MW that showed growth inhibitory activity against the pathogenic microorganism *S. aureus* MRSA and in different experiments, high glucose concentration (80 g/L) did not inhibit its growth although exopolymer production was observed (data not shown). Whereas, *Pseudoalteromonas haloplanktis* growing in a culture with a high concentration of casamino acids (100 g/L) as nitrogen and carbon sources limited the final cell density (Wilmes *et al*., 2010). Then, based on the C/N-ratio of its original habitat and knowing that high glucose concentration did not inhibit the growth of *Pseudoalteromonas* sp., the C/N-ratio range for this work was established.

The effect of the variation in the C/N-ratio of the culture medium on the antimicrobial activity of crude extract and the growth of *Pseudoalteromonas* sp. was studied. In Fig. 1, it is observed that in a glucose-ammonium chloride medium, the increase in C/N-ratio (up to 55) seemed to favor the increase in bacterial biomass (2.7 g L^{-1}) but negatively impacted the antibiotic activity (registering 0.8 U of antibiotic activity), which suggested that this event could be a consequence of carbon and nitrogen available for cell growth, while carbon limitation (C/N-ratio 2.75) seemed to stimulate the antimicrobial activity, as an increase in the inhibition growth of *S. aureus* (maximum activity 3.5 U) was observed. This change in the metabolic response could be result of directing the excess nitrogen towards the production of the secondary metabolite. Supporting these results, the antibacterial activity of the marine fungus *Arthrinium c.f. saccharicola* was inhibited in the presence of glucose limitation (2 g/L) (high N/C-ratio condition) as reported by Miao *et al*. (2006). However, results from Lauritano *et al*. (2016), looking for various biological activities produced by microalgae under different culture conditions, showed that when the microorganism grew in an environment with nitrogen limitation (high C/N-ratio condition), it expressed antimicrobial activity against *S. aureus*, among other pharmacological activities. This would also suggest that the influence of the variation in the C/N-ratio on the production of secondary metabolites and cell growth varies between different microorganisms.

Citrate is an abundant ion in nature and some bacteria can use it as a source of carbon and/or energy. The routes of its degradation are well known. To investigate the effect of C/N-ratio in the range of 0.05 to 1 in the culture medium, as well as the nature of the carbon source in the production profile of antimicrobial activity and growth of *Pseudoalteromonas* sp., sodium citrate and ammonium chloride were used as carbon and nitrogen sources respectively. In Fig. 2, the antimicrobial capacity registered maximum activity (2.7 U) at the lowest C/N-ratio (0.05). In addition, the increase in C/N-ratio of the medium with citrate stimulated microbial growth, registering maximum

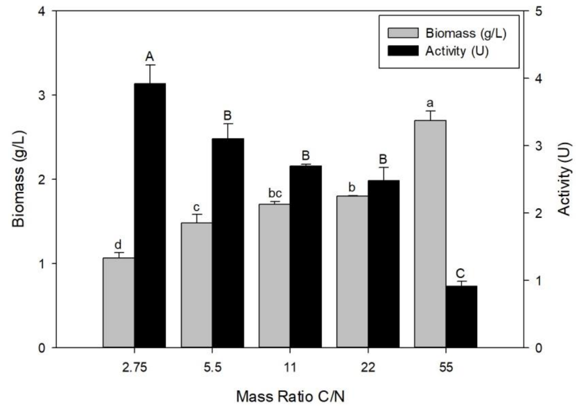

Fig. 1. Effect of the initial C/N-ratio in a medium formulated with glucose and NH_4^+ as a carbon and nitrogen source on biomass production and antibacterial activity from marine *Pseudoalteromonas* sp. Each point represents the mean of three independent experiments with two replicates each ± SD. Letters indicate statistical differences between data means as determined by an ANOVA (P < 0.005).

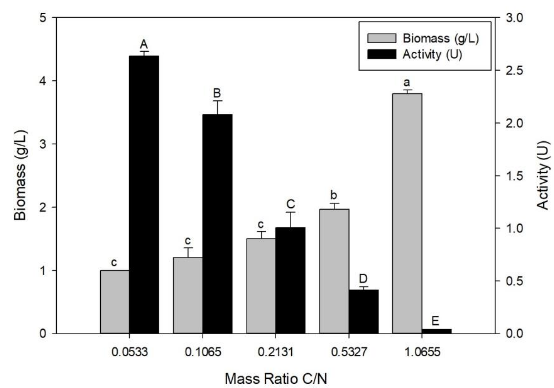

Fig. 2. Effect of the initial C/N-ratio in a medium formulated with citrate and NH_4^+ as carbon and nitrogen sources on biomass production and antibacterial activity from marine *Pseudoalteromonas* sp. Each point represents the mean of three independent experiments with two replicates each ± SD. Letters indicate statistical differences between data means as determined by an ANOVA (P < 0.005).

biomass concentration (4 g L^{-1}, C/N-ratio 1.06) but negatively impacted the antibiotic activity until it was completely suppressed despite the fact that C/N-ratio 1.06 < 3. This could be result of the regulatory effect of the carbon sources that are rapidly assimilated. That is, citrate is an easily metabolizable substrate that can sustain maximum cell growth and inhibit or stimulate secondary metabolism in some bacteria (Sánchez *et al*., 2010; Wang *et al*., 2019). According to these results, studies with the novobiocin-producing bacterium *Streptomyces niveus*, showed that in the culture formulated with citrate and glucose as carbon sources, the aminocoumarin antibiotic was suppressed during the citrate utilization phase (Barberel and Walker, 2000). On the other hand, Yao *et al*. (2020), in experiments with communities of marine bacteria in biofilm models, confirmed that the effect of the addition of sodium citrate was to stimulate biomass production quantitatively in less time than their control without sodium citrate. Thus, the results again suggested that the limitation of the carbon source probably stimulated the antimicrobial activity against *S. aureus*, while the increase in C/N-ratio favored biomass production. However, the observed effect of the C/N-ratio of the medium on the production of antimicrobial activity and growth of *Pseudoalteromonas* sp., seemed to depend on the nature of the carbon source.

Secondary metabolism is generally restricted to the stationary phase and is often considered the result of nutrient limitation. In this order of ideas, the increase of C/N-ratio up to 55 would challenge *Pseudoalteromonas* sp to grow under carbon excess conditions (nitrogen limiting), resulting in a positive correlation between biomass and the C/N-ratio but with reduction of the antimicrobial activity, although not total repression or inhibition. That is, under low nitrogen conditions, growth rate would be expected to decrease and change in a counteracting effect in the metabolism favoring antimicrobial activity. However, the carbon flux was probably directed towards exopolysaccharides (EPS) formation as macroscopic changes in the culture with appearance of viscosity (data not shown). Generally, marine bacteria are capable of producing metabolites such as EPS to protect themselves from osmotic shock, and this would explain the increase in biomass and the active secondary metabolism.

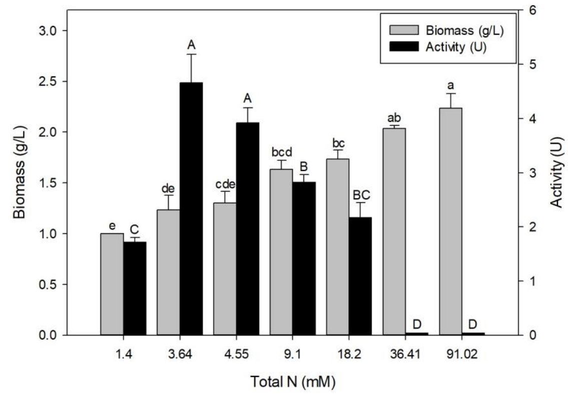

Fig. 3. Effect of the initial total nitrogen concentration of the medium on biomass production and antibacterial activity from marine *Pseudoalteromonas* sp. in a medium formulated with glucose and NH_4^+ as a carbon and nitrogen source. Each point represents the mean of three independent experiments with two replicates each ± SD. Letters indicate statistical differences between data means as determined by an ANOVA (P < 0.005).

3.2 *Effect of total nitrogen concentration in a medium with inorganic nitrogen sources*

The influence of nitrogen availability on secondary metabolism, particularly in the production of nitrogen-containing molecules, is well known. In some cases, the synthesis of secondary metabolites can be suppressed by rapidly assimilable nitrogen sources such as ammonia. In this study, to investigate the effect of the total nitrogen concentration of the medium on the growth and production of antimicrobial activity of *Pseudoalteromonas* sp., C/N-ratio (2.7) was kept constant, and glucose was used as a carbon source and ammonium chloride as a nitrogen source at different concentrations.

The results presented in Fig. 3, indicated that with the increase in the concentration of total inorganic nitrogen in the media, an increase in biomass production was observed. For example, in the case of 91 mM inorganic nitrogen, 2.3 g L^{-1} of biomass was obtained. On the contrary, the production of antibacterial activity tended to increase with the decrease in the concentration of total nitrogen (except with 1.4 mM), observing a maximum antibacterial activity (4.7 U).

When *Pseudoalteromonas* sp. was grown in a nitrogen concentration of 3.6 mM, there was a total suppression of the activity with 36.4 and 91 mM of initial nitrogen of the medium, that is, a condition of nitrogen concentration bigger than 18 mM (Fig. 3). Similar behavior to these results was observed by the group of Gonzáles *et al*. (1994), who reported the effect of ammonium chloride on the formation of gentamicin in *Micromonospora purpurea* where they warned that increasing the concentration of the nitrogen source until 20 mM, stimulated the formation of gentamicin and cell growth, although values bigger than 150 mM decreased significantly both parameters. Likewise, in the bacterium *Pseudomonas MCCB* 103 and in the *fungus Aspergillus fumigatus CY018*, low concentrations of ammonium chloride favored the production of pyocyanin and ketamine, respectively, observing that concentrations above 3.5 g L^{-1} of ammonium chloride had a negative effect on both growths such as ketamine production (Preetha *et al*., 2006; Zhang *et al*., 2016).

On the other hand, Liu *et al*. (2019), studied the effect of the concentration of ammonium acetate on the production of ketamine, chaetominine, produced by the fungus of marine origin *Aspergillus fumigatus CY018* and observed that the optimization of maximum production was obtained by supplying ammonium acetate up to a concentration of 50 mM, and higher concentrations had a negative effect on the production of chaetominine, while the biomass production increased with the increase in the concentration of the nitrogen source. Contrary to these results, the tacrolimus metabolite with immunosuppressive activity produced by *Streptomyces tsukubaensis* improved its productivity under conditions of high C/N-ratio (8.7), observing an effect on tacrolimus production only when the carbon source was modified without observing the same behavior when it was altered the nitrogen source (Moreira *et al*., 2020). Therefore, our results suggested that more than the C/N-ratio, the total nitrogen concentration of the medium (bigger than 18 mM) was the determinant factor in the antimicrobial activity variation of the protein produced by *Pseudoalteromonas* sp. since low C/N-ratio remained constant (C/N-ratio 2.7).

3.3 *Effect of total nitrogen concentration in a medium with organic nitrogen sources*

Numerous studies have shown that rapidly metabolizable nitrogen sources usually decrease the production of antibiotics in different microorganisms. Marine bacteria, in addition to assimilating inorganic nitrogen, also use nitrogen-containing organic compounds for growth and production of secondary metabolites and their incorporation likewise is regulated by the presence of other carbon and nitrogen sources and by the C/N-ratio of the substrates (Kroer *et al*., 1994; Bautista *et al*, 2017; Nofiani *et al*., 2020). Based on our previous studies, the growth and antimicrobial activity of *Pseudoalteromonas* sp. were sensitive to the concentration of amino nitrogen and the type of substrate when the bacteria grew on peptone and yeast extract as carbon and nitrogen sources (Bautista *et al*, 2017). To elucidate whether the increase in the nitrogen concentration in the medium or the ammonium itself affected growth and antimicrobial capacity of *Pseudoalteromonas* sp., the bacterium was cultivated in different concentrations of peptone-yeast extract, keeping the glucose concentration constant at 4.5 g L^{-1}.

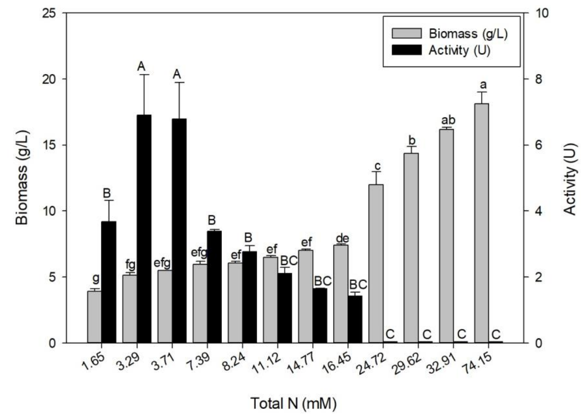

Fig.4. Effect of organic nitrogen source on biomass production and antimicrobial activity from marine *Pseudoalteromonas* sp. in a medium formulated with a mix of yeast extract and peptone as nitrogen source. Each point represents the mean of three independent experiments with two replicates each ± SD. Letters indicate statistical differences between data means as determined by an ANOVA ($P < 0.005$).

As we can see in Fig. 4, in the complex medium there was greater stimulation of biomass production (17 g L^{-1}) than in the medium formulated with ammonium chloride (Fig. 3). While the antimicrobial activity showed the same behavior pattern in both nitrogen sources, also observing a peak of maximum antimicrobial activity (7.5 U) in the cultures containing 3.29 and 3.7 mM of initial total nitrogen, being notable that total suppression of the activity was observed at concentrations bigger than 18 mM as in the ammonium culture of Fig. 3.

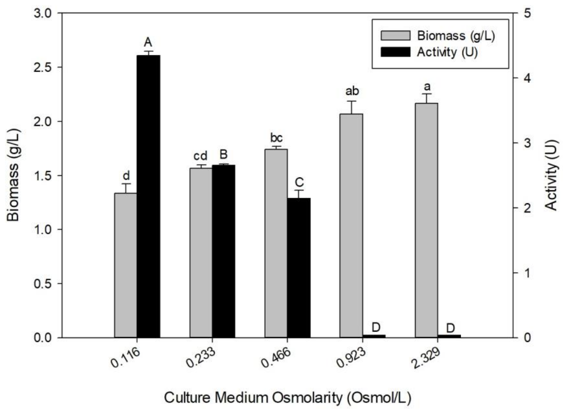

Fig.5. Effect of medium osmolarity on biomass production and antimicrobial activity from marine *Pseudoalteromonas* sp. Each point represents the mean of three independent experiments with two replicates each ± SD. Letters indicate statistical differences between data means as determined by an ANOVA (P < 0.005).

Martínez-Castro *et al*. (2012) conducted an analysis of the effect of peptone-yeast extract and ammonium salts as a nitrogen source in the production of biomass and the immunosuppressant tacrolimus from *Streptomyces tsukubaensis* where they reported that peptone and yeast extract significantly increased cell growth, but completely suppressed tacrolimus production. In addition, ammonia exerted a negative control in the biosynthesis of tacrolimus only at high concentrations (bigger than 2 g L^{-1}). However, the organic nitrogen source does not exert the same effect on secondary metabolism in all bacteria. For example, in the case of the formation of gentamicin in the Gram-positive bacterium *Micromonospora purpurea*, glutamate and glutamine increased the biosynthesis of the secondary metabolite by 65 % and 37 %, respectively, as well as the stimulation of biomass production (Himambindu and Annapurna, 2006).

Consequently, our results suggested that the nature of the nitrogen source significantly affected bacterial growth, while the antibacterial capacity of *Pseudoalteromonas* sp. was affected exclusively by the total nitrogen concentration present in the medium regardless of the chemical nature of the nitrogen compounds used. Then, the antimicrobial activity capacity of *Pseudoalteromonas* sp. was dependent of the quantity available of the nitrogen sources.

3.4 *Effect of the solute concentration of the medium on the antimicrobial activity*

Bacteria in general and marine bacteria in particular, produce and/or incorporate organic and inorganic solutes from the environment to adapt to salinity conditions and balance the external osmotic pressure (Roberts 2005). To analyze the effect of increasing the concentration of solutes, the number of osmoles in the culture medium formulated with different concentrations of glucose and ammonium chloride was calculated. Fig. 5, shows the impact of the osmolarity of the medium on microbial growth and its antimicrobial capacity, observing that 2330 mOsm L^{-1} did not affect the development of microbial growth. However, under conditions of 923 and 2300 mOsm L^{-1} the antimicrobial activity was completely abated.

The marine bacteria members of the genus *Pseudoalteromonas* are considered moderate halophilic bacteria and require Na^+ ions to live. On the one hand, in response to osmotic stress, compatible low molecular weight solutes, such as amino acids, betaine, glycine, etc., accumulate intracellularly to avoid osmotic imbalance that interferes with metabolic reactions (Song *et al*., 2020). On the other hand, the salinity of seawater in the Campeche Sound is between 36.4-34.6 % (Hebbeln *et al*., 2014) with approximate osmolarity of 1200 mOsm L^{-1}, so the observed results of antimicrobial activity in Fig. 5 was probably due to the effect of the total nitrogen concentration (Figs. 3 and 4) and not to the concentration of solutes 923 and 2300 mOsm L^{-1}.

Conclusions

In conclusion, investigating the factors that affect the synthesis and regulation of new antibiotics also involves the search for the optimal culture medium for its production. Our results suggested that more than the C/N-ratio of the medium, one of the main factors that intervene in the regulation of bacterial growth of marine *Pseudoalteromonas* sp was the quantity and nature of available nitrogen sources, while the antibacterial activity of the antimicrobial protein was affected only by the total nitrogen concentration present in the medium regardless of the chemical nature of the nitrogen sources.

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