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COMPARISON OF GROWTH METHODS AND BIOLOGICAL ACTIVITIES OF BRAZILIAN MARINE *Streptomyces*

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Abstract - The present work describes the study of the growth and the cytotoxic and antitumor activities of the extracts of the marine microorganisms *Streptomyces acrymicini* and *Streptomyces cebimarensis,* the latter a new strain. Both microorganisms were collected from coastal marine sediments of the north coast of São Paulo state. Growth was performed in a shaker and in a bioreactor using Gym medium and the broths of both microorganisms were extracted with ethyl acetate and n-butanol. Three extracts, two organic and one aqueous, from each microorganism were obtained and tested for cytotoxic and antitumor activity using the SF-295 (Central Nervous System), HCT-8 (Colon) cell lines, and the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. The growth methods were compared and show that, although the shaker presented reasonable results, the bioreactor represents the best choice for growth of these microorganisms. The biological activity of the different extracts was evaluated and it was demonstrated that the growth methodology may influence the secondary metabolite production and the biological activity.
Keywords: Marine *Streptomyces*; Cultivation; Biological activity.

INTRODUCTION

The chemical investigation of plants and animals (Natural Products Chemistry) has resulted in the discovery of hitherto unknown organic compounds, many of which have been used in pigments, insecticides, drugs, etc. Studies of plants and terrestrial microorganisms have been shown to be extremely important as they generate many economically important organic compounds. For example, 25% of the anticancer drugs in clinical use and approximately 25% of semi-synthetic drugs are derived from natural products [Davidson, 1995].

Marine microorganisms are present in the water column, as well as on the surface of all animate and inanimate objects in the sea. In fact, many previously studied invertebrates presented associated or symbiotic microorganisms, a fact which makes this research area more important, not just from an ecological point of view but also for determining the influence of these microorganisms on the production of secondary metabolites. Marine microorganisms,

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including bacteria, fungi and microalgae, have received increasing attention in recent years and are considered to be a rich and unexplored source of new bioactive metabolites [Davidson, 1995; Fiedler *et al*., 2005; Bull and Stach, 2007].

Bacteria of the order Actnomycetales are common soil habitants and present an unprecedented ability to produce antibiotics. It is estimated that over 50% of the isolated antibiotics are from the genus *Streptomyces* and *Micromonospora*. In general, the actinomicetes are the most valuable from an economic and biotechnological point of view. The actinomicetes are responsible for the production of half of the bioactive secondary metabolites: antibiotics, antitumor agents, immunosuppressive agents and enzymes [Fenical and Jensen, 2006].

The first description of a marine actinomicetes was in 1984 with the discovery of the species *Rhodococus marinonascene* and, since then, various members of the genus *Dietzia*, *Rhodococus*, *Streptomyces*, *Salinispora*, *Marinophilus*, *Slawaraspora*, *Salinibacterium*, *Aeromicrobium marinum*, *Williamsia maris* and *Verrucosiapora* have been isolated from the oceans [Fenical and Jensen, 2006].

Despite the fact that the study of marine actinomicetes for the discovery of new bioactive metabolites is still in the beginning, many new compounds have been isolated by several authors in recent years. Abyssomicin C (Figure 1 (a)) is a polycyclic polyketide compound with antibiotic activity and presents potent activity against Gram-positive bacteria and S*taphilococcus aureus* resistant to vancomycin, and also presents the potential to be developed as an antibacterial agent for bacteria resistant to drugs [Lam, 2006]. Diazepinomicin (ECO-4601) is the only dibenzodiazepinone produced by strains of *Micromonospora* (Figure 1 (b)), posses antibacterial, anti-inflammatory and antitumor activity, in addition to broad spectrum cytotoxic *in vitro* activity and demonstrated *in vivo* activity against glioma, breast cancer and prostate cancer in mice [Lam, 2006]. The preclinical development of Diazepinomicin (ECO-4601) was completed by Ecopia BioSciences Inc. in Canada in 2006 [Lam, 2006].

Salinosporamide A (NPI-0052), a new β-lactone-γ-lactam isolated from *Salinispora tropica* (Figure 1 (c)), is a new proteasome inhibitor which induces apoptosis in multiple myeloma cells with an action mechanism distinct from that of the proteasome inhibitor in clinical use. This compound entered into clinical studies for human cancer treatment in 2006 [Lam, 2006].

In addition to producing Salinosporamide A (NPI-0052), *Salinospora tropica* also produces two macrolides, the Sporolides A (Figure 1 (d)) and B (Figure 1 (e)). These compounds are formed by two different poliketides, containing a large number of oxidized carbons, which contribute to the unusual structures of these compounds. Although they did not present antimicrobial and anticancer activity in the tests realized, these structures show the high potential of marine actinomicetes to produce novel secondary metabolites [Lam, 2006].

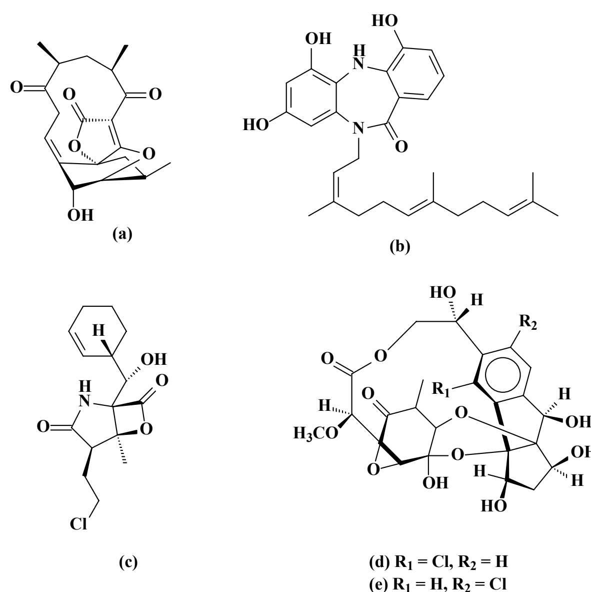


Figure 1: Structures of (a) Abissomicin C, (b) Diazepinomicin (ECO-4601), (c) Salisnosporamide A (NPI-0052), (d) Sporolide A and (e) Sporolide B.

The main purpose of the present work was to study the growth of *Streptomyces cebimarensis* and *Streptomyces acrymicini*, using shaker and bioreactor. It was also the objective of this work to evaluate the cytotoxic and antitumor activity of the extracts provided from both growth methods and compare the growth methodologies employed and the biological activity of the extracts obtained. To the best of our knowledge, there are no reports of chemical studies or biological activity evaluations of the *Streptomyces cebimarensis* secondary metabolites.

EXPERIMENTAL

The strains of marine *Streptomyces cebimarensis* (new strain) and *Streptomyces acrimycini* were collected in São Sebastião, north coast of São Paulo

state [Berlinck *et al*., 2004]. The identification of the strains was performed by Berlinck *et al.* by the analysis of the 16S rDNA sequence indicating that the first strain was a new Streptomycete species [Berlinck *et al*., 2004]. Previously, a comparative growth study with both strains was performed using various cultivation media: A1RDP, ISP4, ISP5 in addition to minimum medium with variations of four different sources of carbon and four different sources of nitrogen, but the best results were obtained with Gym medium. Consequently, both strains were cultivated in a shaker and in a bioreactor using growth medium Gym (2g.L^{-1} $CaCO_3$, 4 g.L^{-1} glucose, 4 g.L^{-1} yeast extract, 10 g.L^{-1} malt extract) and conditions favorable to the microorganisms' growth [Mugnai *et al*., 1989].

The cultivation in a shaker was carried out at 200 rpm and 28 ºC using Erlenmeyers (500 mL) containing 50 mL of the inoculum broth. The inoculum was prepared in three steps: (1) A cryogenic tube, with vegetative microorganism cells (3.5 mL), was transferred to an Erlenmeyer containing the medium (Gym) to perform the reactivation phase. (2) After 24 h, 5 mL was transferred to an Erlenmeyer containing 45 mL of Gym, which was the medium used for growth. (3) After a further 24 h, the inoculums obtained were transferred (5 mL) to a fresh medium (Gym) to perform the production phase. The experiments in the shaker were performed in triplicate. However, the results express the average of the 3 experiments and the difference was under 5%.

Cultivation in the bioreactor was performed in Bioflo II bioreactors, with 4 L of capacity, at 292 rpm and 28 °C. The pH and the dissolved oxygen (DO) were not controlled, just monitored, as the experiment had the goal to simulate the conditions observed in the shaker. The inocula (10%) were prepared in the same way as for shaker growth. The experiments in the bioreactor were done only a single time.

The cellular concentration (Cx) was evaluated by the dry mass of suspended solids. 20 mL of the sample were centrifuged for 15 minutes at 4 °C and 15.500 g and washed two times, re-suspending the precipitate in 20 mL of an acetic acid solution (5%) and a second time in 20 mL of distilled water. The final precipitate mass obtained was maintained at 60 °C for 24 h in a recipient of known mass.

The substrate concentrations of glucose and maltose (Cs) in Gym medium in the experiments in the bioreactor were measured by HPLC (Waters) using a Shodex KS-802 column (Lonpak – Millipore) and water (Milli-Q) as eluent. The equipment conditions were 80 °C and 1 mL.min^{-1}. The detector used was the W410 Refraction index (Waters). The calibration curve was obtained uising solutions of standard compounds (glucose and malt extract).

The broth of both strains was centrifuged to separate the cells. The floating mass was extracted with ethyl acetate and then with n-butanol. From these three extracts of each strain and each growth method, 12 extracts were obtained. The extracts were denoted Sa.1 and Sc.1 and were obtained from the extraction with ethyl acetate. The extracts denoted Sa.2 and Sc.2 were obtained from the extraction with n-butanol. The aqueous extracts were called Sa.3 and Sc.3.

All the extracts were tested in cytotoxic bioassays using the MTT method, which is used by the Screening Program of the *National Cancer Institute* in the USA [Skehan *et al*., 1990]. The bioassays were realized using the SF-295 (Central Nervous System), HCT-8 (Colon) cell lines, given by the National Cancer Institute (USA). All the bioassays were performed in triplicate.

All the cells were cultivated in RPMI 1640, supplemented with 10% fetal bovine serum, 1% of antibiotics. The cells were maintained in a bacteriological oven at 37 °C and a 5% CO_2 atmosphere. The samples were diluted in DMSO (pure and sterile) and were tested at the concentration of 25 µg/mL. The bioassays by the MTT method have been used in the program of screening of the National Cancer Institute (NCI) and it is fast, sensitive and cheap. Basically, the bioassay analyses the viability and the metabolic state of the cell. It is a colorimetric analyses based on the conversion of the salt tetrazolium 3-(4,5-dimethyl-2-tiazol)-2,5-diphenyl-2-H-bromide (MTT) into formazan blue by the mitochondrial enzymes present only in the metabolically active cells. The cells were plated at the concentration of 0.1 x 10^6 cells/mL and were incubated for 72 hours in a bacteriological oven at 37 °C and a 5% CO_2 atmosphere. After this, the cells were centrifuged and the sobrenadant removed; 150 µL of MTT solution were then added and the plates were incubated for 3 hours. The absorbance was measured after dissolution of the precipitate with 150 µL of pure DMSO, in the plate spectrophotometer at 595 nm. The experiments were statistically analysed according to the average ± average standard deviation of the Growth Cell Inhibition using the GraphPad Prism Program. The statistics of growth inhibition are presented in Table 1 as GI% (Growth Inhibition Percentage).

Table 1: Results of the cytotoxic and antitumor activity of the extracts obtained by the microorganism cultivation in the shaker and in the bioreactor.

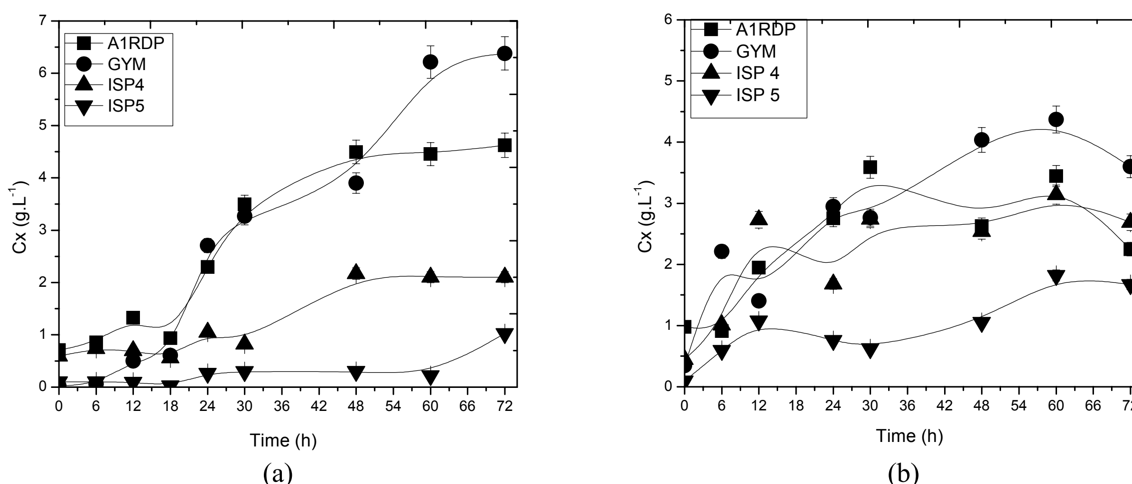
Cultivation	**Cultivation in shaker**					
Strain	***Streptomyces acrymicini***			***Streptomyces cebimarensis***		
Extracts	**Sa-1**	**Sa-2**	**Sa-3**	**Sc-1**	**Sc-2**	**Sc-3**
SF295	95.0 ± 4.75	19.0 ± 0.95	10.0 ± 0.5	55.0 ± 2.75	22.0 ± 1.1	16.0 ± 0.8
GI%	0.5 ± 0.02	7.0 ± 0.35	6.0 ± 0.30	5.0 ± 0.25	7.0 ± 0.35	5.0 ± 0.25
HCT-8	92.0 ± 4.6	20.0 ± 1.0	17.0 ± 0.85	76.0 ± 3.8	16.0 ± 0.8	18.0 ± 0.9
GI% *	2.0 ± 0.1	0.2 ± 0.1	7.0 ± 0.35	2.0 ± 0.1	3.0 ± 0.15	6.0 ± 0.30
Cultivation	**Cultivation in bioreactor**					
Extracts	**Sa-1**	**Sa-2**	**Sa-3**	**Sc-1**	**Sc-2**	**Sc-3**
SF295	101.0 ± 5.05	13.0 ± 0.65	65.0 ± 3.25	48.0 ± 2.4	103.0 ± 5.15	41.0 ± 2.05
GI%	1.0 ± 0.05	2.0 ± 0.1	10.0 ± 0.5	6.0 ± 0.30	1.0 ± 0.05	2.0 ± 0.1
HCT-8	98.0 ± 4.9	68.0 ± 3.4	34.0 ± 1.7	23.0 ± 1.15	26.0 ± 1.3	25.0 ± 1.25
GI% *	5.0 ± 0.25	3.0 ± 0.15	2.0 ± 0.1	6.0 ± 0.30	8.0 ± 0.4	5.0 ± 0.25

*GI% Growth Inhibition Percentage

RESULTS AND DISCUSSION

The marine microorganism strains *Streptomyces acrimycini* and *Streptomyces cebimarensis* were collected in São Sebastião, north coast of São Paulo state [Berlinck *et al*., 2004]. Both strains were deposited in the Culture Collection of CPQBA-UNICAMP and in the DZMC – German Collection of Microorganism and cells. To the best of our knowledge, until now there are no studies on the isolation, characterization or biological activity evaluation of the *Streptomyces cebimarensis* secondary metabolites.

As explained in the Experimental section, previously a comparison of various growth media was performed using A1RDP, ISP4, ISP5 in addition to minimum medium with four different sources of carbon and four different sources of nitrogen. The cellular concentrations of *Streptomyces acrymicini* e *Streptomyces cebimarensis* strains are represented in Figure 2 (a) and (b), respectively. Both strains did not develop well in ISP5 medium and had a better growth in Gym medium, reaching approximately 4 $g.L^{-1}$ in 48 hours of cultivation. In the case of *Streptomyces acrymicini,* growth was greater than 6 $g.L^{-1}$. Comparing the results obtained in cultivation, both strains showed similar results in terms of increased cell concentration with the four media described, assimilating glucose and maltose and starch (A1RDP) as the source of carbon, resulting in the best results for biomass increase. Regarding the adequacy of the nitrogen source, organic nitrogen sources favored the growth in contrast to inorganic sources.

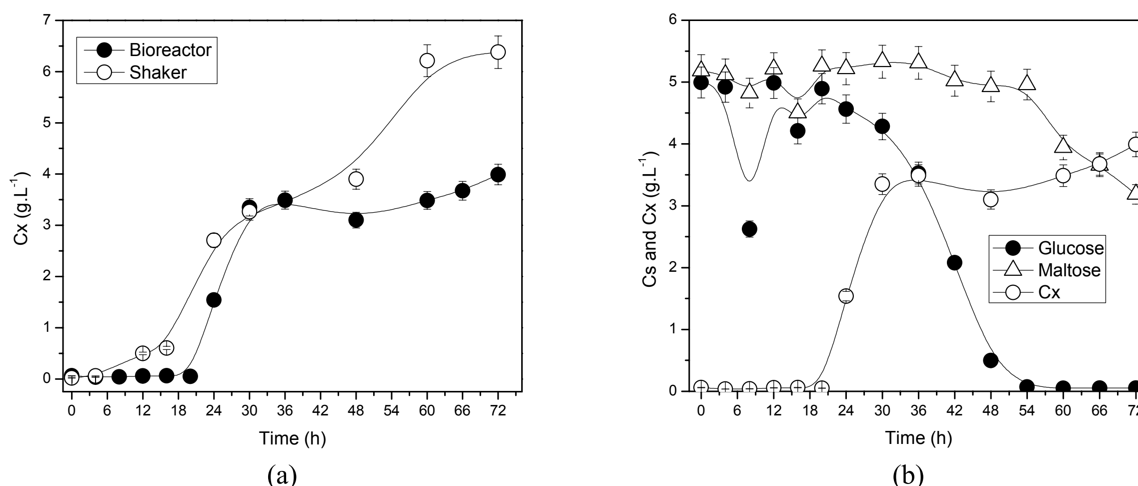


(a) (b)

Figure 2: (a) Growth profile of *Streptomyces acrymicini* in the media A1RDP, Gym, ISP4 and ISP5; (b) Growth profile of *Streptomyces cebimarensis* in the media A1RDP, Gym, ISP4 and ISP5.

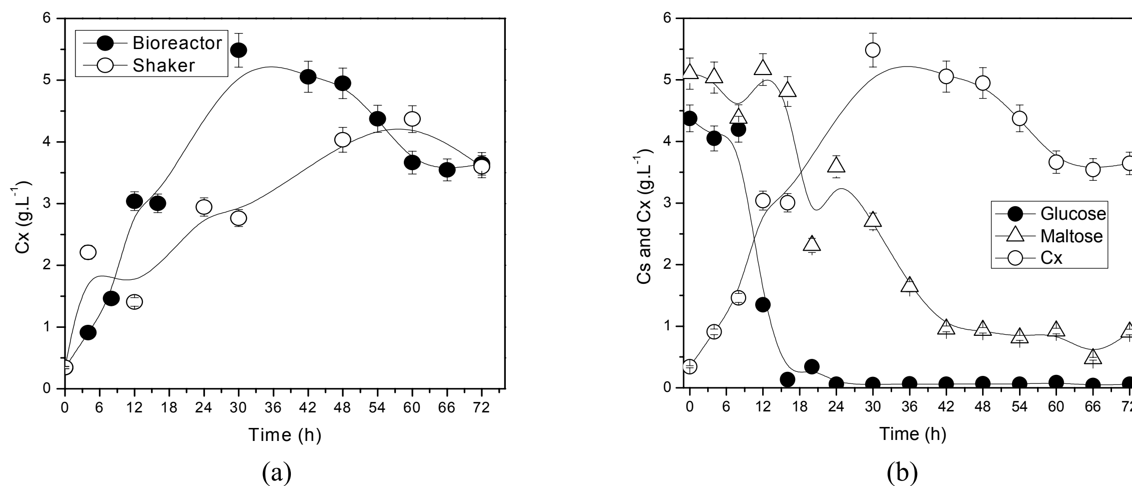
The growth evolution and the substrate and cell concentration for both strains can be seen in Figures 3 (a) and 4 (a). In the growth study of both marine actinomicetes strains reasonable growth results were observed in both the shaker and bioreactor. In the shaker *Streptomyces acrymicini* and *Streptomyces cebimarensis* produced a Cx of 6.38 g/L in 72 h of cultivation and 4.37 g/L in 60 h, respectively. In the bioreactor a Cx of 3.99 g/L was obtained in 72 h of cultivation for *Streptomyces acrymicini* and 5.48g/L in 30 h of cultivation for *Streptomyces cebimarensis*. It was verified that higher growth for *Streptomyces cebimarensis* occurred in the shaker and the higher cellular mass increase for *Streptomyces acrymicini* occurred in the bioreactor cultivation; in this case no limitation by dissolved oxygen (DO) was observed. The concentration of Dissolved Oxygen (DO) was not inferior to 30%.

In the bioreactor it was possible to follow the consumption of carbon sources. Both marine strains demonstrated a typical behavior of a diauxic micro-organism, consuming the glucose first and then the maltose. It was possible to note that both marine strains consume, preferentially, glucose as the carbon source and, when the glucose is finished, both strains consume maltose. It was also verified that, in the bioreactor cultivation of *Streptomyces acrymicini,* glucose takes 54 hours to be totally consumed (Figure 3 (b)), while *Streptomyces cebimarensis* consumes all glucose in 24 hours of cultivation and, after that, the strain maintains its growth with maltose (Figure 4 (b)).



(a) (b)

Figure 3: (a) Evolution of the growth in the shaker and in the bioreactor using Gym medium for *Streptomyces acrymicini* at 28 °C; (b) Substrate concentration (Cs) (Glucose and Maltose) and Cellular concentration (Cx) time profiles, cultivations in the bioreactor of *Streptomyces acrymicini* at 28 °C.



(a) (b)

Figure 4: (a) Evolution of the growth in the shaker and in the bioreactor using Gym medium for *Streptomyces cebimarensis* at 28 °C; (b) Substrate concentration (Cs) (Glucose and Maltose) and Cellular concentration (Cx) time profiles, cultivations in the bioreactor of *Streptomyces cebimarensis* at 28 °C.

It is important to note that in both *Streptomyces acrymicini* cultivation methods there was a large *lag* phase (adaptation and preparation of the micro-organism to growth) starting with an exponential phase at more than 12 hours of shaker cultivation and approximately 20 hours in bioreactor cultivation. This fact is apparently not related to the carbon source availability, because there is still glucose until 54 hours of bioreactor cultivation and only then is the maltose consumed.

The *Streptomyces cebimarensis* strain rapidly consumes all the glucose (20 hours) during its logarithmic phase of growth. Even when the maltose starts to be consumed the strain follows its exponential growth demonstrating that the lack of glucose does not impede this phase to proceed until 30 hours of cultivation.

All the extracts were tested in bioassays of cytotoxic activity by the MTT method [Skehan *et al*., 1990]. As can be seen in Table 1, the cytotoxic activity of the extracts obtained from the shaker and bioreactor cultivations present considerable differences. While for the shaker cultivation of *S. acrimycini* only the ethyl acetate extract (Sa-1) presented activity in both tested strains (SF295 and HCT-8), for the bioreactor cultivation all the extracts were active. Considering the microorganism *S. cebimarensis*, for the shaker cultivation only the ethyl acetate extract (Sc-1) presented activity in both tested strains (SF295 and HCT-8), while for the bioreactor cultivation only the butanol extract (Sc-2) was selectively active for SF295 cells.

The results obtained show that the cultivation conditions influence directly in the secondary metabolite production. Probably the stress to which the microorganism is submitted is different in the cultivation methods used in this work and it contributes to the production of different secondary metabolites.

For *Streptomyces acrimycini*, shaker cultivation favored the production of low polarity metabolites bioactive for Central Nervous System cells and for Colon cells, i.e., these metabolites were only localized in the non-polar extract (Sa-1, ethyl acetate extract). In contrast, for the *Streptomyces acrimycini* bioreactor cultivation, the bioactive metabolites were dispersed, presenting non-polar, medium polarity and polar compounds. The butanol extracts concentrated bioactive compounds selective for Colon cancer cells, the water extract concentrated bioactive compounds specific for Central Nervous System cells and the non-polar extract concentrated bioactive compounds for both strains tested. Thus, it is possible to verify that the cultivation in the bioreactor is better for *S. acrimycini*.

Taking into account the marine microorganism *Streptomyces cebimarensis*, the shaker cultivation promoted the production of nonpolar compounds bioactive for Central Nervous System and Colon cell strains (extract Sc-1, ethyl acetate), while the bioreactor cultivation produced bioactive medium polarity compounds selective for Central Nervous System cells (extract Sc-2, butanol).

CONCLUSIONS

An increasing number of papers in Microorganism Natural Products describe the isolation of new bioactive metabolites and many of them are in advanced clinical tests. The importance of such studies at a national and international level is therefore clear.

It is possible to conclude that the cultivation method reflects directly on the bioactive secondary metabolite production because the biological activities of the extracts from both cultivation methods were different. It is also possible to state that the cultivation in the bioreactor enables control of the cultivation conditions, which favored the growth of the microorganism in addition to the production of a larger amount of crude extract, which is better for isolatation of the bioactive compounds.

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