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THE EFFECT OF THERMIC PRETREATMENT AND ANTIBIOTICS ON THE SELECTIVE ISOLATION OF THE CULTURABLE ACTINOMYCETES FROM THE ALGERIAN DESERT SOIL

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Résumé

Dans le but de définir les conditions d'isolement des actinomycètes à partir des sols désertiques, les sols de la ville de Biskra située à la porte du désert Algérien ont été analysés. Deux types d'échantillons ont été préparés. Le premier a été chauffé à 110°C pensant 10 minutes, l'autre n'a subit aucun traitement. L'isolement a été effectué à partir de ces deux types de sols, sur le milieu amidon caséine additionné d'une combinaison de plusieurs agents antifongiques et antibactériens. Cinq différents milieux d'isolement ont été également testés dans cette étude. Les résultats montrent que le traitement du sol à la chaleur n'est pas recommandé. En effet, le nombre des actinomycètes isolés été de 38. 10^4 ufc.g^{-1} quand les échantillons de sol sont chauffés et de 92.10^4 ufc.g^{-1} sans chauffage. Le prétraitement thermique réduit donc les actinomycètes à plus de 50%. L'antifongique et l'antibactérien qui permettent l'élimination des germes indésirables, sont la nystatine et l'acide nalidixique à des concentrations respectives de 50 µg/ml et 10 µg/ml. D'après ces résultats, le milieu d'isolement le plus favorable pour les actinomycètes des sols désertiques de la région étudiée est le glucose-yeast-malt (GLM).

Mots cles : *Actinomycètes, sol désertique, prétraitement thermique, milieu sélectif.*

Abstract

In order to define the isolation conditions of actinomycetes from desert soils, the soils of the town of Biskra, situated at the gate of the Algerian Sahara was analyzed. Two types of samples have been prepared; the firt one was heated up at 110 °C during 10 minutes and the second did not undergo any heat processing. The isolation was carried out from these two types of soils on the starch casein medium added to a combination of several antifungal and antibacterial agents. Five different isolation mediums have been tested in this study. The results showed that the pretreatment of the soil with heat is not recommended. Indeed, the number of isolated actinomycetes was 38. 10^4 cfu.g^{-1} when the soil samples were heated up and 92.10^4 cfu.g^{-1} when no heating was used. The thermic processing reduces then the actinomycetes by more than 50 %. The antifungal and antibacterian which permit the elimination of undesirable germs are the nystatin and the nalidixic acid with respective concentrations of 50 µg/ml and 10 µg/ml. According to these results, the most favorable isolation medium for actinomycetes of desert soils studied is Glucose-yeast extract-malt (GLM).

Keywords: *Actinomycetes , Desert soil , Thermic pretreatment, Selectif medium*

ملخص

من أجل تحديد ظروف عزل الأكتينوميسات من التربة الصحراوية، تم تحليل تربة من مدينة بسكرة و التي تقع في مدخل صحراء الجزائر. تم إعداد نوعين من العينات. العينة الأولى تم تسخينها على درجة حرارة 10 م لمدة 10 دقائق و العينة الثانية لم تخضع لأي علاج. تم عزل الأكتينوميسات من هاتين العينتين على البيئة الغذائية نشاء كازيين مضاف إليه مجموعة من المضادات البكتيرية و الفطرية. لقد تم كذلك في هذه الدراسة اختبار خمس بيئات غذائية. أظهرت النتائج أن معاملة التربة بالحرارة غير مستحسن، حيث عزل عدد 38. 10^4 ufc.g^{-1} من التربة المعاملة بالحرارة و 92.10^4 ufc.g^{-1} من التربة الغير مسخنة. يظهر على ضوء ه النتيجة أن معالجة التربة بالحرارة تحفظ عدد الأكتينوميسات إلى أكثر من 50٪ . المضادات البكتيرية و الفطرية تسمح بإزالة الجراثيم الغير مرغوب فيها هي النيستاتين و حمض الناليديكسيك بقيم 50 ميكروغرام /مل و 10 ميكروغرام/مل على التوالي. حسب هذه النتائج فإن بيئة العزل الأكثر ملائمة للأكتينوميسات الموجودة في التربة الصحراوية للمنطقة المدروسة هو الجلوكوز خميرة الشعير (GLM .

الكلمات المفتاحية *: الأكتينوميسات , التربة الصحراوية علاج حراري , بيئة انتقائية*

The actinomycetes are the most researched microorganisms because of their capacity to produce primary and secondary metabolites which are essential for health, such as antibiotics [19, 5].

The selective isolation of the actinomycetes from their habitat constitutes a problem. Indeed, too many substracts favor fungi and bacteria with a rapid growth and prevent an easy isolation of the actinomycetes which have a generation time relatively long [29].

Several techniques have been used in the selective isolation of actinomycetes. They are essentially based on the processing of samples, the development of appropriate medium and the addition to isolating mediums of the inhibiting substances, stopping the growth of the invading germs [16, 21, 13, 10].

The processing of samples applied in different screening programs of the actinomycetes which produce antimicrobials agent tent to favor the growth of these bacteria with respect to the other bacteria and fungi [26]. The heating of soil samples is one of the most used processing techniques. A temperature of 55 °C or 100 °C during one hour reduces considerably the number of bacteria and fungi without affecting the number of actinomycetes [9]. In other work, the soil samples are treated at a temperature of 110 °C during 10 minutes [1]. Other pre-treatments at heat have been used as well by some reseachers for selective isolation of actinomycetes from non desertic soils [25, 18, 15].

Some soils treated at 120 °C during 60 minutes have been used for the selective isolation of actinomycetes belonging to the genus of *Microbispora* and *Streptosporangium* [17]. These techniques of thermic processing use the high resistance of actinomycete arthrospores against the dryness and temperature conditions [17, 25, 26, 7].

The presence in the isolation medium of the chitin, starch, glycerol, arginine, asparagin, casein and the nitrate, leads to a selective isolation of the actinomycetes, whereas, the bacteria and fungi grow weakly [29]. The use of agar with parafin favors the isolation of strains belonging to the *Nocardia* genus [7].

The cycloheximide (actidione) at 50-100 $\mu g.ml^{-1}$, the pimaricin at 10 $\mu g.ml^{-1}$ and the nystatin at 50 $\mu g.ml^{-1}$ concentration, are the most used antifungals, as additives, in the isolation mediums of actinomycetes [8, 24]. The most used antibacterians in the isolation of actinomycetes are polymixin at 5 $\mu g.\ ml^{-1}$ and penicillin at 1 $\mu g.ml^{-1}$ [29, 7]. The nalidixic acid is an antibacterian acting against bacteria with a negative Gram coloration. It has been successfully used at a concentration of 10 $\mu g.ml^{-1}$ mixed with the nystatin and the actidione respectively at 25 and 10 $\mu g.ml^{-1}$ in a screening of actinomycetes from sediments of bay [28].

Research works concerning the isolation of actinomycetes from arid soils are rare [11, 21, 32, 6, 4]. To the best of our knoroledge, the study of the optimal conditions which provide the isolation of these micro-organisms from arids soil samples are more rare in the literature [12, 20, 2]. These conditions are of capital importance and increase the success of this research by multiplying the chances of isolating these microorganims [12, 27, 2].

The goal of this study consists of defining the effect of thermic pretreatment of the soil samples and to establish the role of some antibacterial and antifungal in improving culture conditions and isolation of actinomycetes. We also test some actinomycetes isolation media, favourable for the isolation of these bacteria from samples of arid soils. In our work, we have tested, desert soils coming from the region of Biskra town situated at the gate of the Algerian Sahara.

MATERIALS AND METHODS

Sampling and processing

Three soil samples are collected at 4 km away from Biskra longitude 005E44, Latitude 034N51. 100 to 150 g of soil are collected according to the technique of Pochon and [23]. After removing the first five centimeters of the superficial layer of the soil. Each sample is devided into two lots of 50 g. One of these two lots undergoes a heating at 110 °C during 10 min [1]. 1g of each soil heated or not is introduced into a tube containing 10 ml of sterile physiological water (NaCl 9 $g.l^{-1}$) and sterile glass balls of 2 mm diameter. The samples are vigorously mixed during several minutes. The suspensions obtained constitute the solutions to analyse.

Tested antibiotics

The starch casein medium ajusted at pH 7.0 [10] and added to the antibiotic concerned has been used as a basic medium.

The amphotericin B, the penicillin and the polymyxin B are dissolved in sterile distilled water. The antibiotics not soluble in water like the nystatin and the nalidixic acid

are put in suspension in ethanol in such a way that the final concentration of the solvent does not exced 1 ml per litre of medium [5].The solutions are prepared at the moment of usage and are mixed to the medium in surfusion at 45 °C, according to the following combinations:

-Medium without antibiotics

-Medium+nystatin at 50 µg.ml^{-1}

-Medium+amphotericin B at 50 µg. ml^{-1}

-Medium+nystatin and amphotericin B at 50 µg. mL^{-1} each

-Medium+nalidixic acid at 10 µg.ml^{-1}+nystatin at 50 µg. ml^{-1}

-Medium+nalidixic acid at 10 µg. ml^{-1}+amphotericin B at 50 µg. mL^{-1}

-Medium+nalidixic acid at 10 µg. ml^{-1}+amphotericin B+nystatin at 50 µg. ml^{-1} each

-Medium+polymyxin B sulphate at 5 µg. ml^{-1} + sodium penicillin at 1 µg. ml^{-1} +nystatin and amphotericin B at 50 µg. ml^{-1} each

The isolation mediums

The actinomycetes are isolated and counted on the most used mediums for the selective isolation of this type of microorganism from the soil. The composition of the mediums is as follows:

-Glucose-yeast extract-malt (GLM) (3 g.l^{-1} yeast extract (Merck), 3 g.l^{-1} malt extract (Merck), 5 g.l^{-1} peptone (Merck), 10 g.l^{-1} glucose (Merck), 20 g.l^{-1} agar (Merck), pH 7.2).

-Bennett (1 g.l^{-1} yeast exract (Merck), 1 g.l^{-1} beef extract (Merck), 2 g.l^{-1} casaminoacide (Merck), 10 g.l^{-1} glucose (Merck), 15 g.l^{-1} agar (Merck), pH 7.3).

-Water-yeast extract-agar (WYE) modified by Reddi and Rao (0.25 g.l^{-1} yeast extract (Merck), 18 g.l^{-1} agar (Merck), pH 7.2).

-Glucose-asparagin (10 g.l^{-1} glucose (Merck), 0.5 g.l^{-1} asparagin (Prolabo, Paris, France), 0.5 g.l^{-1} KH_2PO_4, (Merck), 15 g.l^{-1} agar (Merck), pH 7.3).

-Pridham medium (10 g.l^{-1} glucose (Merck), 4 g.l^{-1} yeast extract (Merck), 10 g.l^{-1} malt extract (Merck), 20 g.l^{-1} agar (Merck), pH 7.3). All these mediums have the nalidixic acid at 10 µg.ml^{-1} + nystatin at 50 µg. ml^{-1} added to them.

Dilutions and sowing

Decimal dilutions in 9 mL of physiological water (NaCl 9 g.l^{-1}) have been realised. 1 ml of each dilution (10^{-1} at 10^{-7}) is sowed in surface; three plates are sowed by dilution and incubated at 28 °C during 21 days.

Enumeration of colony

The colony of actinomycetes counted on Petri plates are those which present macroscopic characteristics of Actinomycetale bacteria. The observations are carried out after 21 days of incubation. The confirmation of the filamentous aspect is obtained by microscopic observation after Gram coloration. The bacterian colony which does not present the aspect of actinomycete are counted after 48 h of incubation, then observed in a microscope after Gram coloration. The colonies of fungi are recognised after observation under binoculars and under an optical microscope, then, they are counted.

RESULTS AND DISCUSSION

The results gathered in table I indicate that with the processing of the samples with heat, the number of non filamentous bacteria is 40.10^4 cfu.g^{-1}, against 74.10^4 cfu.g^{-1}, that is almost the double of the case when the samples are not processed. All bacteria which grew up are in majority with a positive Gram coloration. The number of yeasts is also reduced by half when the samples are not processed with heat. However, the moulds are not sensitive to this type of processing. The heating of soil samples shackle the development of non filamentous bacteria and yeasts and has no effect on moulds.

The use of a basis medium without antibiotics and without a processing with heat permitted the isolation of 9.10^4 cfu.g^{-1} actinomycetes against 5.10^4 cfu.g^{-1} using the same medium without antibiotics and with thermic processing (table 1).

The total number of *Actinomycetale* bacteria is 38.10^4 cfu.g^{-1} when the soil samples are heated up and 92.10^4 cfu.g^{-1} without heating (table 2). The processing with a heat has reduced then at 50 % the number of actinomycetes isolated from this type of sample. From the results obtained, we observe that the processing with heat decreases by half the indesirable germs but acts also, in the same manner against the actinomycetes. These results

agree perfectly with those obtained by Nonomura and Ohara, 1969 who showed that the processing with heat is used only for selective isolation of some types of actinomycetes whereas the *Streptomyces* do not resist to this type of processing.

This is explained by the fact that some spores of *Streptomyces* do not resist to temperatures over 50 °C in humid heat and to 70 °C in dry heat [16]. In addition, the actinomycetes in the mycelium growing stage cannot resist to the heat conditions imposed during the thermic processing of samples [28].

The nalidixic acid inhibate the bacteria with negative Gram coloration, when it is used with all the isolation mediums tested reduces slightly the number of bacteria other than the actinomycetes in comparison with mediums without nalidixic acid and with or without processing with heat. The bacteria which grew are all with a positive Gram coloration.

On the other hand, in the mediums where the nystatin was added, the number of isolated actinomycetes increases in the presence of the nalidixic acid by 18.10^4 cfu.g^{-1} colony to 21.10^4 cfu.g^{-1} without processing of samples with heat and 4.10^4 cfu.g^{-1} colony to 8.10^4 cfu.g^{-1} when the samples are treated with heat.

Contrarily to mediums containing the amphotericin B, the number of isolated actinomycetes is practically unchanged (table III). In soils not procesed with heat and in the presence of nalidixic acid, the number of isolated actinomycetes is 41.10^4 cfu.g^{-1} and only 14.10^4 cfu.g^{-1} of non filamentous bacteria. In absence of nalidixic acid the number of isolated actinomycetes is 37.10^4 cfu.g^{-1} against 40.10^4 cfu.g^{-1} non filamentous bacteria (table 3). In this study, the combined effect of non processsing with heat and the use of nalidixic acid give better results.

The two antifungals tested in this work, are the nystatin and the amphotericin B at concentration 50 µg.mL^{-1} each. According to the results obtained, gathered in table I, the starch casein medium added to nystatin inhibates the fungi present in these soil samples and allows the growth of 1.10^4 cfu.g^{-1} of yeasts only among 11.10^4 cfu.g^{-1} present in the medium without antifungals taken as witness.

The amphotericin B inhibates totally the filamentous fungi present and allow, however, the isolation of 4.10^4 cfu.g^{-1} of yeasts. The mixture of these two antifungals acts in the same manner as the nystatin alone. The actidione (cyloheximide) has not been used in this study because of its high toxicity.

The use of the starch casein medium added to polymyxine B sulphates at 5 µg.ml^{-1}, sodium penicillin at 1 µg.ml^{-1} and the nystatin + amphotericin B at 50 µg.ml^{-1} each allows the isolation of a very reduced number of bacteria, yeast and indesirable fungi (table 1). However, the isolation of actinomycetes from this medium is not satisfactory; only 8.10^4 cfu.g^{-1} actinomycetes have been isolated from soil samples, processed with or without heat against 22.10^4 cfu.g^{-1} from the same basic medium added to nystatin (table 2).

The views concerning the use of antibacterians are contreversed. According to some authors, most of antibacterials agents used inhibate a lot of actinomycetes [24,18, 15]. On the other hand, other chearchers recommend the use of a mixture of antifungals and antibacterials agents for the isolation of actinomycetes [8, 29, 3, 30].

Several studies showed that the use of antibacterians inhibates the *Streptomyces* [16, 7] and leads to the selective isolation of some genus of actinomycetes only [14, 22, 26, 31].

The isolation carried out on five mediums chosen from the literature, showed that the medium which favor the isolation of an important number of actinomycetes from arid soils of this region is the Glucose-yeast extract-malt (GLM) (table IV). This complex medium is composed of glucose as a source of carbon.

Therefore, it looks like the majority of the tested mediums in this study. However, its composition is different from other mediums by the presence of an important variety of organic azote sources as malt extract, yeast extract and peptone. The other mediums offer equally well an important Actinomycetale flora. Consequently, they should not be dismissed in this type of screening.

Table 1: Effect of processing with heat (10 min at 110 °C) of soil samples on the isolation of on filamentous bacteria, yeasts and fungi.

	With processing with heat			Without processing with heat		
Medium	Number of non filamentous bacteria	Number of yeast	Number of fungi	Number of non filamentous bacteria	Number of yeast	Number of fungi
B med+nyst	5[(a)]	0	0	12	1	0
B med+amph B	8	2	0	18	2	0
B med+nyst+amph B	6	0	0	10	1	0
B med+na+nyst	3	0	0	3	0	0
B med+na+amph B	5	1	0	6	1	1
B med+na+nyst+amph B	2	0	0	5	0	0
B med+polyB+pen+ nyst+amph B	2	0	0	1	1	0
B med	9	3	3	19	8	2
Total	**40**	**6**	**3**	**74**	**14**	**3**

(a): Mean number, repetition=3 at dilution 10^{-4}
B med= Basic medium, nyst=nystatin, amph B= amphotericin B, na= nalidixic acid, poly B= polymixin B, pen= penicillin.

Table 2: Effect of processing with heat on the isolation of actinomycetes of the desert soil of Biskra.

	with processing with heat	without processing with heat.
Medium	Number of actinomycetes colony (dilution10^{-4})	Number of actinomycetes colony (dilution10^{-4})
Med + nyst.	4 [(a)]	18
Med+ amph B.	4	13
Med+ nyst+ amph B.	6	6
Med + an + nyst.	8	21
Med + an + amph B.	3	12
Med + an + nyst + amph B.	5	8
Med + poly B + peni + nyst + amph B.	3	5
B med	5	9
Total	38	92

(a): Mean number, repetition=3
B med= Basic medium, nyst=nystatin, amph B= amphotericin B, na= nalidixic acid, poly B= polymixin B, pen= penicillin.

Table 3: Combined effect of processing with heat and the nalidixic acid on the isolation of actinomycetes and other bacteria of the desert soil of Biskra.

	With processing with heat				Without processing with heat			
	na +		na -		na +		na -	
Medium	Actino	Bact	Actino	Bact	Actino	Bact	Actino	Bact
B med+nyst	8[(a)]	3	4	5	21	3	18	12
B med+amph B	3	5	4	8	12	6	13	18
B med+nyst+amph B	5	2	6	6	8	5	6	10
Total	**16**	**10**	**14**	**19**	**41**	**14**	**37**	**40**

(a): Mean number, repetition=3 at dilution 10^{-4}
B med= Basic medium, nyst=nystatin, amph B= amphotericin B, na= nalidixic acid.

Table 4: Number of isolated actinomycetes on isolation medium added to the nalidixic acid at 10 $\mu g.ml^{-1}$ + nystatin at 50 $\mu g.ml^{-1}$ and without processing with heat.

Isolation medium	Mean number of actinomycetes ($cfu.g^{-1}$)
Glucose-asparagin	$1.8 . 10^5$ [(a)]
Pridham	$3 . 10^5$
GLM	$9 . 10^5$
Bennett	$4 . 10^5$
WYE	$0.03 . 10^5$
Starch casein	$2.1 . 10^5$

(a) : Mean number, repetition=3

CONCLUSION

The selective isolation of actinomycetes from arid soil samples coming from the region of Biskra is favoured by some conditions.

The processing of soil samples with heat is not recommended because it reduces considerably the actinomycete flora.

The introduction of antibacterian substances with large spectrum as sodium penicillin and polymyxin B sulphate in isolation mediums inhibates the undesirable bacteria but acts negatively on some actinomycetes.

The use in the isolation mediums of the nalidixic acid at 10$\mu g.ml^{-1}$ and the nystatin at 50 $\mu g.ml^{-1}$ allows the total elimination of fungi and most of the yeasts and bacteria with negative Gram, present in this type of sample. The (GLM) medium gives a high number of actinomycetes colonies. Thus, we preconise in a study of actinomycete isolation from desertic soils samples like those coming from the region of Biskra, the use of a rich medium like a GLM medium added to the nalidixic acid at 10 $\mu g.ml^{-1}$ and the nystatin at 50 $\mu g.ml^{-1}$.

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