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Evaluation of culture media for *Paenibacillus larvae* applied to studies of antimicrobial activity

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ABSTRACT

This study was conducted to compare different liquid culture media for *Paenibacillus larvae* growth in order to find the best one to be used in studies on activity of antimicrobial substances, such as essential oils. *P. larvae* presented poor growth in usual broths such as Mueller-Hinton, commonly employed in antimicrobial activity assays. Growth in liquid media was evaluated using *Paenibacillus larvae* strains isolated from hives located in different geographical zones. The MYT medium (Mueller-Hinton broth, yeast extract and thiamine) was selected out of the eight liquid media analyzed, as it proved to be the most adequate due to its higher absorbance at 620 nm. The following mean values were obtained from the four *P. larvae* strains: 0.227 ± 0.016 for the Cobo strain, 0.279 ± 0.015 for La Plata strain, 0.758 ± 0.020 for Mechongué strain and 0.244 ± 0.0079 for Sierra de los Padres strain, respectively.

Key words: antimicrobial activity, culture media, *Paenibacillus larvae*

RESUMEN

Evaluación de medios de cultivo para el crecimiento de *Paenibacillus larvae* aplicables en estudios de actividad antimicrobiana. Este trabajo está orientado a comparar diferentes medios de cultivo líquidos para el crecimiento de *Paenibacillus larvae.* El objetivo fue encontrar el más apropiado para utilizar en estudios de actividad antimicrobiana de diferentes sustancias, tales como aceites esenciales. *P. larvae* presenta un crecimiento débil en medios de cultivo como el Mueller-Hinton, comúnmente usado en ensayos de actividad antimicrobiana. Se evaluó el crecimiento en caldos de cultivo de cepas aisladas de colmenas ubicadas en diferentes zonas geográficas. De los ocho medios analizados, el MYT (Mueller-Hinton, extracto de levadura y tiamina) mostró ser el más apropiado, en éste se observó el mayor valor de absorbancia a 620 nm. Los valores obtenidos en promedio para los cuatro aislamientos de *P. larvae* evaluados fueron 0,227 ± 0,016 (cepa de Cobo); 0,279 ± 0,015 (cepa de La Plata); 0,758 ± 0,020 (cepa de Mechongué) y 0,244 ± 0,0079 (cepa de Sierra de los Padres).

Palabras clave: actividad antimicrobiana, medios de cultivo, *Paenibacillus larvae*

American Foulbrood (AFB) is one of the most widespread and destructive brood diseases which affects the honey bee larval stage (*Apis mellifera).* The causative agent is *Paenibacillus larvae* (7), a gram-positive and spore forming bacterium that infects queen, drone, and worker larvae alike. Adult bees, however, are not affected by AFB.

The growth of this bacterium occurs in two forms: vegetative (rod-shaped bacterial cells) and spores. Only the spore stage is infectious to honey bees. The spores germinate into the vegetative stage soon after they enter the larval gut, and continue to multiply until larval death. AFB is one of the few bee diseases capable of killing a colony. The prevention and control of this disease have features of its own, as the spores can remain viable for long periods and survive under adverse environmental conditions (10, 11).

While *P. larvae* sporulates and multiplies efficiently in the hemolymph of bee larvae, most strains grow poorly in or on artificial media. Different culture media have been developed based on larval debris, honey, wax and adult bees in order to isolate *P. larvae*. Several of them were especially evaluated with the purpose of analyzing the spore recovery of this species from honey, not only for examining their composition but also the antibiotic addition, and the assessment of different incubation conditions. The MYPGP developed by Dingman and Stahly (4) and made up of yeast extract, Mueller-Hinton broth, glucose, K_2HPO_4, sodium pyruvate and agar, yielded the highest percentage of spore recovery; while J agar, brain heart agar fortified with thiamine, Columbia agar supplemented with ovine blood, and agar supplemented with horse blood proved to be less efficient in this respect (12). Continued researches have also been underway to optimize culture conditions so as to obtain maximum *P. larvae* recovery from honey. To this end, different concentrations of nalidixic and pipemidic acids antibiotics

were tested. The conclusion drawn was that $MYPGP_{NALPIA}$ B (9 µg/ml of nalidixic acid and 20 µg/ml of pipemidic acid) is the most appropriate medium for honey spore recovery within heterogeneous populations. Conversely, and in connection with vegetative cell cultures, the most appropriate media would be MYPGP and $MYPGP_{NALPIA}$ (6 µg/ml of nalidixic acid and 10 µg/ml of pipemidic acid) (13). The AFB spore isolation from adult bees (9) employed a J medium with 3 µg/ml of nalidixic acid, and (5) used MYPGP agar supplemented with the same amount of antibiotic.

Yet, further research should be conducted on liquid media so as to favor the evaluation of vegetative cell growth of this microorganism by turbidimetry (4, 8), and, at the same time, the analysis of antimicrobial activity by serial dilution. There are no recommendations available by NCCLS regarding *P. larvae* CIM determination; and, particularly, this bacterium finds it difficult to grow in Mueller-Hinton liquid broth (6), the usually employed medium in assays of this sort. In earlier studies on the antimicrobial activity of antimicrobial substances, such as essential oils, in which serial dilution methods with BHIT, Mueller-Hinton broth, and Mueller-Hinton broth with thiamine were employed, several problems were encountered. The bacterial growth obtained was non-reproducible among isolations for the first medium and poor for the last two ones, which hindered the observation of the inhibition resulting from the antimicrobial substances, and, hence, the minimal inhibitory concentration measure.

The aim of this work was to obtain a suitable medium applicable to studies of antimicrobial substances, such as essential oils, against *P. larvae*.

Bacterial strains of *P. larvae* were isolated from honeycombs exhibiting clinical symptoms of American Foulbrood. These hives were located in the outskirts of the cities of Mechongué, La Plata, Cobo and Sierra de los Padres, all of them located within Buenos Aires province. Isolation was achieved on MYPGP agar supplemented with 9 µg/ml of nalidixic acid in order to inhibit the growth of *Paenibacillus alvei*. Plates were incubated under microaerobic conditions (5-10% of CO_2), and the strains were identified by employing standard biochemical tests (2). The different strains of *P. larvae* were stored at –80 °C on liquid MYPGP with 15% v/v of glycerol until use.

Vegetative cells of *P. larvae* were grown on MYPGP agar incubating 48 h at 36±0.5 °C under aerobic conditions. Afterwards, they were suspended in double distilled sterile water and standardized to a turbidity level approximating 10^7-10^8 cells/ml (absorbance of 0.258 at a wave length of 620 nm) measured with a spectrophotometer (Bausch & Lomb Spectronic 20, USA).

For the measure of cell growth by optical density (OD) at 620 nm, vegetative cells in liquid media were monitored by reading turbidity at 620 nm (3) with a spectrophotometer UV-VIS Metrolab 325 digital. One ml of the standardized microbial suspension was added to different kinds of media. Sterile media without microorganisms served as control. The liquid media used was Mueller-Hinton broth supplemented with different additives. Concentration selection of all the components in each media was made in relation to Mueller-Hinton labels, Dingman and Stahly (4), and Gochnauer (8).

All the liquid media used and their respective composition were identified by their initials 1) M: 0.2 % of Mueller-Hinton broth dissolved in water; 2) M+T: 0.2% of Mueller-Hinton broth supplemented with 0.1 mg/l of thiamine (autoclaved separately); 3) M+Y: 0.2% of Mueller-Hinton broth, 1.5% of yeast extract; 4) MYT: 0.2% of Mueller-Hinton broth, 1.5% of yeast extract supplemented with 0.1 mg/l of thiamine; 5) MYPGP: 1% of Mueller-Hinton broth, 1.5% of yeast extract, 0.2% of glucose, 0.3% of K_2HPO_4 and 0.1% of sodium pyruvate; 6) MYPGP+T: 1% of Mueller-Hinton broth, 1.5 % of yeast extract, 0.2% of glucose, 0.3% of K_2HPO_4 and 0.1% of sodium pyruvate supplemented with 0.1 mg/l of thiamine; 7) MYPGP without pyruvate: 1% of Mueller-Hinton broth, 1.5% of yeast extract, 0.2% of glucose and 0.3% of K_2HPO_4; 8) MYPGP without glucose: 1% of Mueller-Hinton broth, 1.5% of yeast extract, 0.3% of K_2HPO_4 and 0.1% of sodium pyruvate. Triplicate analyses of each medium and strains were utilized to determine the tests. The media were incubated at 35±0.5 °C, for 24 hours.

Absorbance data from culture cells were statistically analyzed as a randomized complete block design fitting a model: media + strain and its interaction using SAS PROC MIXED (SAS OnlineDoc (rtm). Version 8, Copyright (c)2000, SAS Institute Inc). Least square means were compared using the Tukey-Kramer test. The level of probability used to assess statistical significance was $\alpha = 0.05$.

The pH measurements of MYPGP liquid medium, MYPGP liquid medium without sodium pyruvate and MYPGP liquid medium without glucose were evaluated in order to establish whether the absence of one of their components influenced on pH and, hence, on cell growth.

As shown in Table 1, acceptable growth was observed on MYT broth, with major mean values of optical density, compared to the other liquid medium, with the exception of the Cobo strain. No significant differences were noticed between MYT and the second medium with greater absorbance values, except for the Cobo strain. For all strains, poor growth in the MYPGP medium with thiamine was observed in comparison with the MYPGP medium alone; yet no significant differences ($p < 0.05$) between these two media were noticed for all cases.

The MYPGP medium with no pyruvate yielded turbidimetry values of high absorbance when compared to those of the MYPGP liquid medium, even though these values showed no significant differences for all cases. The broth without glucose showed lower absorbance values (Table 2).

Table 1. Microbial growth expressed as optical density in different liquid media used

	COBO	LA PLATA	MECHONGUÉ	SIERRA DE LOS PADRES
M	0.131 ± 0.016 C	0.072 ± 0.018 A	0.505 ± 0.020 B	0.135 ± 0.0097 A
M+T	0.012 ± 0.016 C	0.085 ± 0.015 A	0.620 ± 0.020 B	0.108 ± 0.0079 A
M+Y	0.266 ± 0.016 A	0.094 ± 0.015 B	0.670 ± 0.020 C	0.224 ± 0.0079 D
MYT	0.227 ± 0.016 C	0.279 ± 0.015 A	0.758 ± 0.020 B	0.244 ± 0.0079 A
MYPGP	0.197 ± 0.019 C	0.278 ± 0.015 A	0.742 ± 0.020 B	0.236 ± 0.0079 A
MYPGP+T	0.174 ± 0.016 C	0.276 ± 0.015 A	0.710 ± 0.020 B	0.230 ± 0.0079 A

Values represent means of optical density (OD) (by triplicate analyses for medium and strains) ± standard error.
Means ± standard error followed by the same letter are not significantly different ($p < 0.05$).

Table 2. Comparison between microbial growth in MYPGP liquid medium and same medium without glucose and pyruvate against four *P. larvae* strains.

	COBO	LA PLATA	MECHONGUÉ	SIERRA DE LOS PADRES
MYPGP [5]	0.276 ± 0.024 A	0.310 ± 0.017 A	0.683 ± 0.0042 B	0.267 ± 0.0087 A
MYPGP without pyruvate	0.285 ± 0.024 A	0.334 ± 0.017 A	0.784 ± 0.0042 B	0.308 ± 0.0087 A
MYPGP without glucose	0.188 ± 0.024 A	0.218 ± 0.017 A	0.519 ± 0.0042 B	0.204 ± 0.0087 A

MYPGP: Mueller-Hinton broth 1%, yeast extract 1.5 %, K_2HPO_4 0.3%, glucose 0.2%, sodium pyruvate 0.1%.
Values represent optical density (OD) means (by triplicate analyses of medium and strains) ± standard error.
Means ± standard error followed by the same letter are not significantly different ($p < 0.05$).

The pH values corresponding to the four strains studied in the different media were measured, i.e MYPGP, MYPGP without pyruvate and MYPGP without glucose. Mean values were 6.11 for all the strains in the first broth, 6.14 for the second broth, and 6.422 in the MYPGP broth for the MYPGP broth without glucose addition.

The indispensable components for *P. larvae* growth are thiamine (B_1 vitamin), yeast extract and several peptones. Sporulation is promoted when glucose and sodium pyruvate are added to the medium (4). Yeast extract provides great variety of nitrogen organic constituents capable of covering the general nitrogen needs and growth factors usually required for microorganism growth (14).

For all of the cases, poor growth was noticed in the MYPGP liquid media with thiamine when compared to MYPGP liquid media. Most likely, this is due to the addition of this micronutrient concurrently with the nutrients of the MYPGP liquid media. The additives can become either growth inhibitors or toxic when their concentration is high (14). Assays on the MYPGP liquid media without glucose and without pyruvate in comparison with the MYPGP liquid media were carried out afterwards (Table 2), so as to explain the lower absorbance detected in the liquid MYPGP in comparison with MYT (Table 1). Findings were consistent with the results published by Dingman and Stahly (4), who stated that pyruvate would inhibit growth and promote sporulation while glucose would favour growth. The growth in these media was evaluated separately, as it can be inhibited by organic acids resulting from glucose fermentation. After these experiments, we tested the selected MYT liquid medium in assays with essential oils against strains of *P. larvae*. For all the experiments, the MYT liquid medium yielded good bacterial growth. Besides, the MIC values in studies of antimicrobial activity could be determined by turbidimetry.

In view of the values of absorbance at 620 nm observed in the MYT broth, which are above those of the MYPGP liquid, it is concluded that the best media to evaluate antimicrobial activity is this new enriched broth. Given its nutritional quality, it favours good *P. larvae* growth; and due to its translucent coloration, it enables bacterial growth assessment by turbidity.

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