Contribution to the determination of the halophilic bacteria content of mineral and biologic media.

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The determination of halophilic bacteria content in biologic and mineral media like muds, soils, salts or preserved foods still remains a delicate technique. Application by different laboratories does not always give corresponding and reproducible results. A comparative study of different current or published methods, available in the scientific literature or commonly carried out in laboratories, allows to identify the relative effects of parameters which characterize the most usual ones. An appropriate standardization, ratified by a brief statistical study, leads to improve both their precision and repeatability.

1. INTRODUCTION

Salt-loving (i.e. halophile) microorganisms do not possess the ability to grow in the absence of salt. Therefore they thrive worldwide in high density saline environments, especially natural salt lakes [1], salterns and saltworks ponds [2]. Due to accumulation within their cells of some pigments like carotenoids and ruberins, they are mainly responsible for the typical reddish color of salterns and salt lakes.

They are able to live in such salty niche thanks to a fascinating biological adaptation including the endurance to osmotic stress, the specific composition of the cell wall, the development of enzymatic activities consistent with high ionic strengths and the presence in some members of a unique, retinal-based protein pigment, bacteriorhodopsin [3].

Most authors [4] recognize four different types of Halophiles: (i) marine or slightly halophiles that exhibit significant growth up to 1 M NaCl (5.5% NaCl w/w) (ii) moderate halophiles that grow better in media from 0.5 to 2.6 M NaCl (2.8-13% NaCl w/w), (iii) average halophiles that develop commonly between 1.6 and 4.2 M NaCl (8.5-19.5% NaCl w/w) and (iiii) extreme halophiles that show optimal growth in media containing 2.5-5.2 M NaCl (12.75-23.3% NaCl w/w). The latter are also known to survive in salts [5] and in heavily salted products like salted fish [6] and cured hides [7].

The red-colored bacteria of the genera *Halobacterium* and *Halococcus* have been considered the dominating types of concentrated brines in marine salterns.

According with this both biological diversity, occurences and specific metabolism, isolation and enumeration of the extremely halophilic bacteria in mineral or biological media remain a delicate technique.

Many efforts have been made from decades [8] to adapt conventionnal microbiological methods to specific requirements of halophilic bacteria [5, 9]. In spite of this, the application by various laboratories do not always lead pertinent and reproducible results.

So in the present work, we try at first to clarify the relative importance of the parameters which characterize the various more commonly practised methods of isolation and enumeration of extreme halophiles.

Then the standardization of the trial sampling protocol is proposed and the influence of this standardization is at least experimentally investigated regarding the repeatability and the precision of the enumeration of Halophilic Bacteria carried out by the Dussault-Lachance method [10].

2. METHODS OF ISOLATION AND ENUMERATION OF HALOPHILIC BACTERIA

The investigated protocols result from publications on the microbiological studies of cured hides [11] and salted fish [12, 13, 14], on the direct microbiological studies of natural brines, saltworks sediments [15] and raw salts [16], on the research for isolation of microorganisms from ancient salt formations [17] and finally on the more fundamental studies of the so particular biology and ecology of the halophilic bacteria [18].

Unfortunately, the descriptions of methods published in most of this scientific literature do not always completely exhibit some characteristics of operating modes, in particular with regard to the followings:

- expression of salt content (units),
- preparation of samples (weight of sample, salt content of dissolving and diluting solutions),
- dilution before plating,
- colonies culture conditions (temperature, growth time).

In this way, parts of these data are often missing in the « Materials and Methods » chapter of the publications. However, when they are available, significant variations are noticed as in Table 1 below which exhibits the characteristics of 6 commonly used methods.

2.1. Expression of salt content

First of all the salt added in the different bodies concerned by this microbiological approach is expressed in specific units that do not correspond to the real value. Like that, the conventional indication of *percent of NaCl (weight to volume* or *w/v*) refers to the preparation mode of the body rather than to its physical composition.

That's why we may encounter simply *« Sodium Chloride - 20% solution »* [19], or more explicitly *« 10% brine (10 g NaCl in 100 ml water) »* [20], whereas these two solutions present a real salt content of respectively 16.67% and 9.09% by weight of the whole resulting body.

Accordingly, assuming the figures in % w/v are appropriate for serial volumic dilutions but do not fit with physical and biological aspects of salt content, all the values cited in the present paper were also systematically converted in percentage by weight (% w/w) each times it was possible [see Table 1]. This allows us to validly compare the various methods as regards the NaCl content of dissolving and diluting solutions as well as of growth medium.

Table 1
Comparison between the main characteristics of current methods for the determination of halophilic bacteria

Origin	Lab. A	Nakashizuka	Dussault	Magnùsson	Canada	Lab. B
Reference	[21]	[22]	[10, 20]	[23]	[19]	[13, 24]
Trial sample dissolution						
Sample weight	10 g	10 g	10 g	11 g	10 g	N.A.
Dissolving volume	90 ml	60 ml	100 ml	99 ml	100 ml	N.A.
Dissolving medium (w/v)	0.5% salt	tap water	10% salt	15% salt	20% salt	20% salt
NaCl contents (w/w) during sample dissolution [a]						
at the beginning	0.5%	0%	9.09%	13.04 %	16.67%	16.67%
at the end	10.4%	14.28%	16.9%	21.06%	23.5%	N.A.
Dilution, plating and culture						
Dilution medium (w/v)	0.5% salt	25% salt	20% salt	15% salt	20% salt	20% salt
NaCl in diluent (w/w)	0.5%	20.0%	16.67%	13.04%	16.67%	16.67%
NaCl in growth medium (w/w)[a]	15.75%	21.9%	15.6%	15.6%	18.1%	18.4%
Growing temperature	20°C	35°C	37°C	35°C	35°C	36°C
Growing length	5 days	21 days	7-10 days	14 days	10-14 days	14 days

[a] Calculated true values according to available data in original paper - N.A.: not available data

2.2. Salinity during initial dissolution

As we can see in Table 1, commonly used dissolving media show a wide gradient of salinities ranging from tap water to near saturated brine.

In this way the microrganisms may be fiercely, and of course adversely for salt-loving ones, placed in very low concentrations of salt at the initial step of sample preparation [21, 22].

According with the fact that tap water is obviously *the absolute poison* for halophiles, the beginning of dissolution in such media should lead to massive disappearing among halophilic organisms present in the sample being dissolved.

2.3. Salinity of the dilution solution

Table 1 shows that salt content in dilution medium ranges from 0.5% to 20% w/w. In addition with final low salt content of dissolving medium, this may also contribute to maintain the microorganisms in a too hyposaline environment with the same adverse effects.

2.4. Salinity of the growth medium

Salt content in growth medium varies from 15.6 to 21.9% w/w and seems to be in a quite good agreement with optimum conditions needed by the most frequently encountered halophilic genera e.g. *Halobacterium* and *Halococcus* (17 to 21% w/w) [25].

2.5. Growing temperature and length

With one exception (Lab. A), all the methods take in account the optimal requirements of halophilic bacteria with regard to the temperature incubation (from 30°C up to 50°C) and to the long length of cultivation requisite by the development of identifiable colonies on agar in Petri dishes [25].

In conclusion, the parameters of the protocols which may mainly influence the results of the enumeration appear to be: (i) preparation of the samples for plating (mass of the sample, salt content of the solutions used for the dissolution and the dilutions), (ii) conditions of the culture of colonies (salt in growth medium, temperature, length).

3. QUANTITATIVE EXPERIMENTS

3. 1. Materials

For the experiments, samples of brines and salts come from Mediterranean and Atlantic coast saltworks. To improve availability of halophilic bacteria, sampling was carrying out preferably from concentrated brines and crushed freshly harvested salt.

3. 2. Method

The method of isolation and enumeration of Extremely Halophilic Bacteria is directly derived from the technique proposed in 1952 by Dussault and Lachance[10] and consisting of surface growth on solid media. Usually called DL-Agar, this technique is always at present routinely used for the determination of red halophilic bacteria contents in curing salts and salted fish [23].

3. 2. 1. Medium

$MgSO_4.7H_2O$	5 g
$Mg(NO_3)_2.6H_2O$	1 g
$FeCl_3.7H_2O$	0.025 g
Proteose peptone	5 g
Glycerol	10 g
Sodium Chloride	200 g
Distilled water	1,000 g

3. 2. 2. Protocol

Of salt, 10 g are dissolved in 100 ml of sterile water with various salt contents. Samples are diluted using 16.7% salt solution (w/w). First dilution is usually 1/100, i.e. 0.1 ml are surface plated on agar. From this dilution further appropriate decimal dilutions are prepared. The plates were incubated for 14 days at 35°C in a saturated atmosphere.

4. ENUMERATION RESULTS

Figure 1 shows the distribution histograms of results of Extremely Halophilic Bacteria enumeration of 60 duplicate samples of salts performed by two different laboratories carrying out the same technique adapted from Dussault-Lachance [10]. It appears clearly that the two laboratories describe the Halophilic Bacteria contents of the examinated salts in a strongly opposite manner with regard to means and dispersion parameters.

The comparison of the protocols exhibited that Lab. D performed the dissolution of samples with a 0.5% salt solution. That's why we evaluate the effect of salinity of dissolving solution on height repetitions of the same salt sample. Figure 2 shows the adverse effect of low salinity during the sample dissolution: from a mean value of 500,000 germs/g,

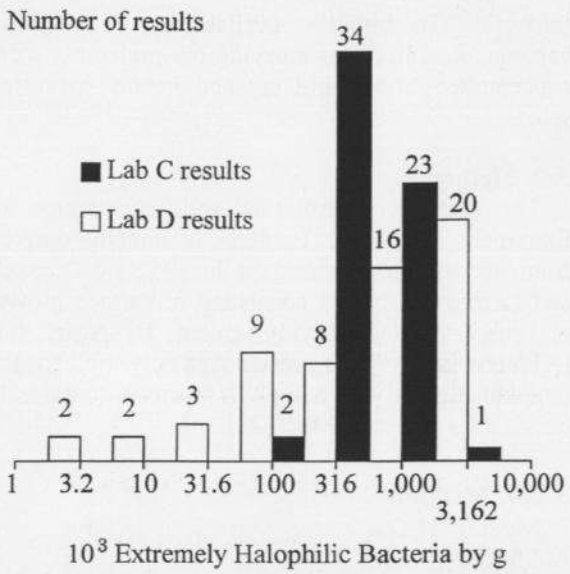

Figure 1. Comparison between results obtained by two laboratories from 60 duplicate samples (see text).

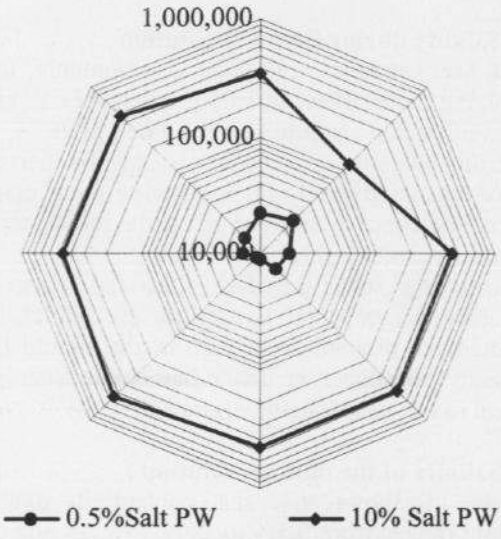

Figure 2. Effect of salinity of dissolving solution on enumeration of EHB (germs/g) - Height repetitions from the same salt sample - PW : Peptone Water.

the *killing* effect of low salt content reduces the halophilic bacteria to less than 30,000 germs/g.

To evaluate this effect during the dilution steps of the protocol, we also compare two salinities (0.5% and 15% salt) of the dilution solution on the same population of 100 samples of salts from various origins. The results, ranged by increasing difference, appear in Figure 3 below. As previously observed, the same *killing* effect of low salt content systematically prevails and affects all the samples with amplitudes that seem to vary almost in proportion with the assumed true values (15% salt).

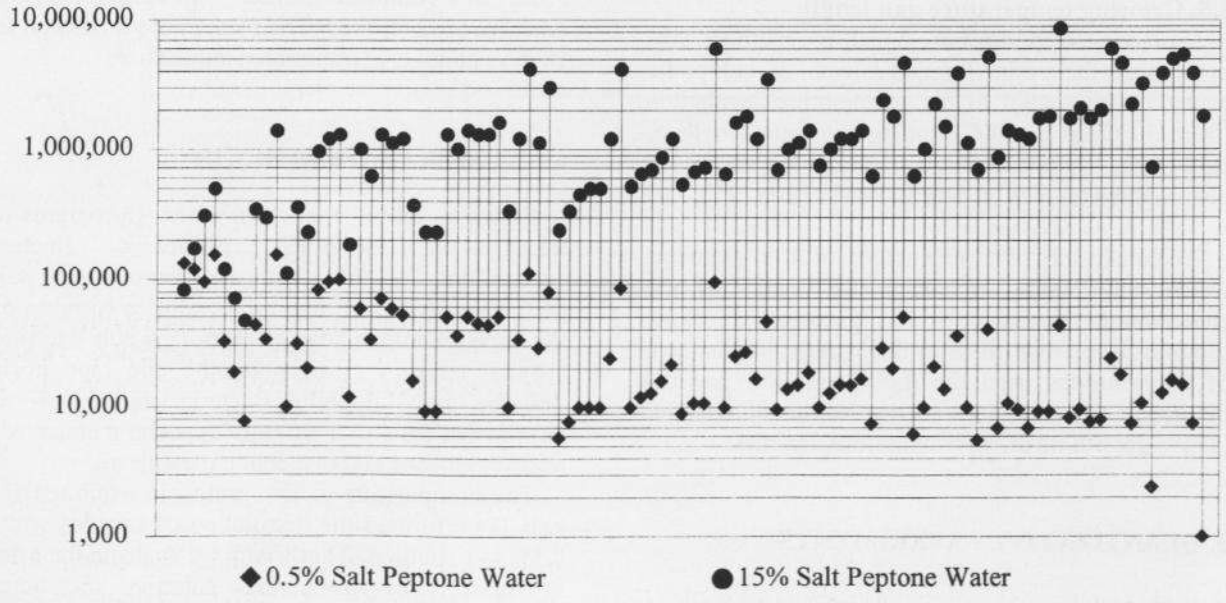

Figure 3. Effect of salinity of dilution solution on enumeration of Extremely Halophilic Bacteria (germs/g) Hundred freshly harvested salt samples.

These features indicate that respect of a minimum salt content in all the liquid media involved along the protocol is a critical point. Thus we propose to standardize the salinity of these media by the followings:

- Use a 16.7% salt solution (w/w) as dissolving and diluting solution (e.g. add 200 g of salt to 1000 g of water).
- Use a 10 g trial sample of salt to dissolve in 250 ml of solution as above. The final salt content will be 19.5% (w/w).

By these means the salt content in the serial dilutions will become again constant at 16.7% (w/w) from the third decimal dilution.

Precision and repeatability are then really improved as we can see in Figures 4, 4 bis and 5.

To verify the effect of standardization we repeat 28 times the enumeration from the same initial dilution of two salts with different Halophilic contents. The Figure 4 and Figure 4 bis shows the distribution diagrams with basic dispersion parameters. In both cases the shape of the distribution is close to Gaussean curve and the coefficient of variation is less than 20% which points out a fair good result for such type of microbiological determination.

At least the complete protocol was tested by ten repetitions of two different samples of salt. Figure 5 beside exhibits the results. The quite circular lines that link the ten observed values give evidence for a good accuracy and repeatability of the method.

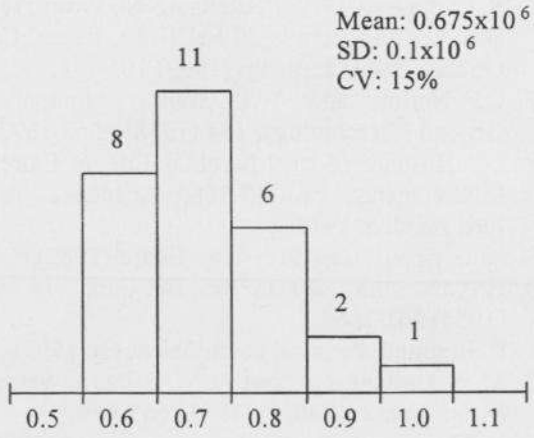

Figure 4. Effect of proposed standardization on enumeration of Extremely Halophilic Bacteria (germs/g) - 28 repetitions of the same sample trial dilution.

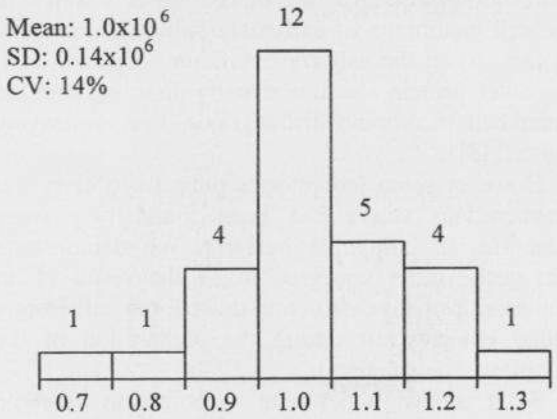

Figure 4 bis. Effect of proposed standardization on enumeration of Extremely Halophilic Bacteria (germs/g) - 28 repetitions of the same sample trial dilution.

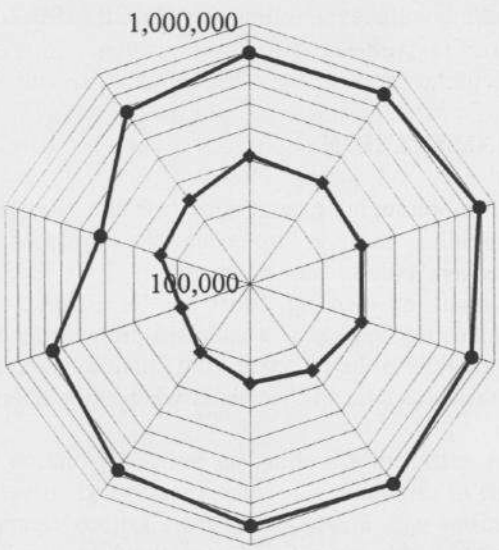

Figure 5. Effect of proposed standardization on enumeration of Extremely Halophilic Bacteria (germs/g) - Ten repetitions of complete protocol for two different salt samples.

5. DISCUSSION

By these results we pointed out that a highly saline environment is essential to maintain the viability of halophilic microorganisms during all the successive phases of the protocol of isolation and enumeration. It is obviously in perfect concordance

with the specific structure of coating or « wall » of the cell membrane of extremely halophilic bacteria; in fact, when the salt concentration drops too low, the outer protein « wall » actually dissolves and the inner cell membrane disintegrates, thus destroying the cell [3].

However some laboratories pursued to carry out enumerations where had been found very weak quantities of halophilic bacteria. We demonstrate that such values appeared to be the result of an important cellular destruction due to an insufficiently salted environment during the preparation of the samples.

So it is always pertinent to point out here the advice of Gibbons [5] who wrote thirty years ago « that in any work on the extreme halophiles one must think *salt* at all times ». It must be also stressed that the extreme halophiles will not survive exposure to salt concentrations of less than 15% even for short period. Thus the proposed standardization maintains a salinity above 16.7% salt in all the stages of the operating protocol; it also allows to minimize the handling volumes as well as to reduce the dissolution time.

6. CONCLUSION

The biologic peculiarities of the Extremely Halophilic Bacteria influence their methods of detection, culture and enumeration. It is therefore essential for insuring count results accuracy and repeatability to respect a standard protocol as close as possible to the effective vital conditions required by these bacteria in the media where they naturally live.

A sufficient sampling, an initial dissolution in a brine of minimum salt concentration and successive dilutions with a solution of same salt concentration constitute the main factors involved in a reliable carrying out of the Halophilic Bacteria enumeration.

By this method, repeatability and precision are then improved as well as the productivity of the analytical operations.

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