MaCuMBA

Marine Microorganisms: Cultivation Methods for Improving their Biotechnological Applications

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Contents

Summary

In this deliverable we present experiments that aimed at improving cultivation conditions for bacterial strains representing new taxa. Cultivation conditions were improved to better screen antitumor activity or to get more biomass for strain characterization purposes.

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1- Improved cultivation conditions for novel groups of deep-sea Actinobacteria

PharmaMar (PhM) has been designing media for the isolation of new deep-sea bacteria. Of a total of 1678 isolates, only 45 failed to produce 16S rRNA PCR products. All other strains were identified based on their 16S rRNA sequence (EzTaxon database, www.ezbiocloud.com). 1520 strains showed >98.5% 16S rRNA gene similarity with 357 described species while 113 strains (9%) displayed <98.5% 16S rRNA gene similarity with known species and may represent new bacterial species (Table 1).

Table 1. List of potential new species of deep-sea bacteria

#(*)	Nearest neighbor	#	Nearest neighbor	#	Nearest neighbor
1	*Agromyces bauzanensis*	**3**	***S. aurantiogriseus***	1	*Streptomyces marinus*
1	*Bacillus benzoevorans*	**14**	***S. aureoverticillatus***	1	*Streptomyces mayteni*
1	*Bacillus foraminis*	**2**	***Streptomyces avermitilis***	1	*S. monomycini*
1	*Bacillus rigui*	1	*Streptomyces bluensis*	**2**	***S. nanshensis***
1	*Defluviimonas denitrificans*	1	*Streptomyces bobili*	1	*S. olivaceiscleroticus*
1	*Glycomyces arizonensis*	**3**	***Streptomyces cacaoi***	**2**	***Streptomyces orinoci***
1	*Isoptericola nanjingensis*	**3**	***Streptomyces calvus***	1	*S. phaeochromogenes*
1	*Kitasatospora arboriphila*	1	*Streptomyces cellostaticus*	1	*S. prasinopilosus*
1	*Micromonospora pisi*	1	*S. diastatochromogenes*	1	*Streptomyces pratensis*
1	*Nitratireductor aquibiodomus*	1	*Streptomyces ederensis*	1	*S. qinglanensis*
1	*Nocardia beijingensis*	**2**	***Streptomyces emeiensis***	**3**	***Streptomyces scabiei***
1	*Nocardia flavorosea*	1	*S. enissocaesilis*	**2**	***S. scabrisporus***
4	***Nocardia niigatensis***	1	*Streptomyces flaveus*	**3**	***S. spectabilis***
6	***Nocardiopsis trehalosi***	**3**	***S. griseoaurantiacus***	**6**	***S. tsukubaensis***
1	*Nonomuraea endophytica*	**5**	***Streptomyces haliclonae***	**2**	***S. variegatus***
1	*Pseudonocardia alaniniphila*	1	*S. hiroshimensis*	1	*S. viridosporus*
4	***Streptomyces albospinus***	1	*Streptomyces incanus*	1	*S. xiamenensis*
4	***S. antibioticus***	1	*Streptomyces iranensis*	**2**	***S. xinghaiensis***
(*) # Number of OTUs (16S sequences) matching to the same nearest neighbor					

About 1453 of the isolated deep-sea isolates have been cultured for antitumor screening, of which 17% were active. Most of them belonged to *Streptomyces* spp (Figure 1). Other active genera were members of the genera *Streptosporangium, Nonomuraea, Nocardiopsis, Nocardia, Myceligenerans, Micromonospora* and *Actinomadura*. Although other isolates grew well, they did not show antitumor activity under the conditions tested.

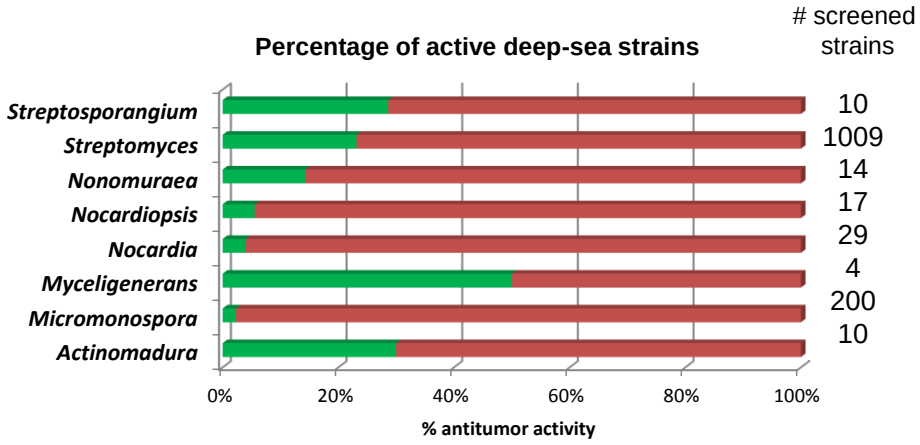

Figure 1. Percentage of strains active for antitumor activity respective to the number screened per genus.

Production media & antitumor activity

Six fermentation conditions (M1-M6) were tested to screen the activity of isolated actinomycetal genera (Table 2). We used our own formulations of production media. M1, M2, M3 were composed mainly of dextrin, dextrose, mannitol, malt extract, peptone, soy flour and marine salts at different concentrations (formulated using Surface Response Optimization algorithms). M1 and M2 contained a high concentration of mannitol (50g/L) and dextrine (68g/L), respectively, while M3 was more balanced. M4 was composed of dried yeast and partially hydrolysed starch (Glucidex >40g/L). M5 was composed mainly of glycerol corn step power, nitrates and marine salts (ASW) at different concentrations. Finally, M6 was a highly complex medium using all M1-M6 ingredients.

Table 2. Percentage of active (antitumor screening) species among within the isolated genera of actinomycetes relative to the test medium.

Genera	M1	M2	M3	M4	M5	M6
Actinomadura	80%	0%	67%	0%	0%	-
Micromonospora	0%	18%	3%	0%	0%	15%
Myceligenerans	0%	100%	100%	0%	0%	-
Nocardia	10%	0%	25%	0%	0%	0%
Nocardiopsis	25%	25%	0%	0%	0%	0%
Nonomuraea	-	-	20%	50%	0%	-
Streptomyces	45%	31%	51%	49%	9%	17%
Streptosporangium	33%	0%	0%	0%	0%	33%

The results for each genus tested were as follows:

- *Actinomadura*: Good production under high concentration of mannitol, but excess of dextrine can be inhibitory of the production of secondary metabolism.
- *Micromonospora*: The best results were obtained using M2 (dextrine) and M6, a high complex media without any nutrient higher than 20 g/L.
- *Myceligenarans*. Clearly production on dextrine with mannitol as inhibitor.
- *Nocardia:* No high concentration of nutrients triggers clearly the cytotoxic activity.
- *Nocardiopsis:* Clearly the biosynthesis of secondary metabolites was related to stressing concentrations of mannitol and dextrine.
- *Nonomuraea*: Best results using starch as carbon source.
- *Streptomyces:* The activity depended on the species rather than the genus. Good results in most cases in general.
- *Streptosporangium*: The excess of mannitol and high complex media were the best scenarii for production.

2- Improved cultivation conditions for a new* Octadecabacter *species

A new *Octadecabacter* strain (NH9_P7) isolated from mussel beds in the Baltic Sea was chosen by DSMZ to improve its cultivation conditions. Although the strain was isolated with Marine Broth medium, all the tests were performed using artificial sea water medium amended with peptone (5 g/L) and yeast extract (1 g/L). To get enough biomass to perform the different tests, the strain was grown for 5 days at pH 7.3, 15°C, and 2.3% NaCl.

To determine its optimum pH, the strain was grown at different pHs at 20°C and growth was followed by measuring absorbance at 600nm. The specific growth rate µ were calculated with the R software (Figure 2).

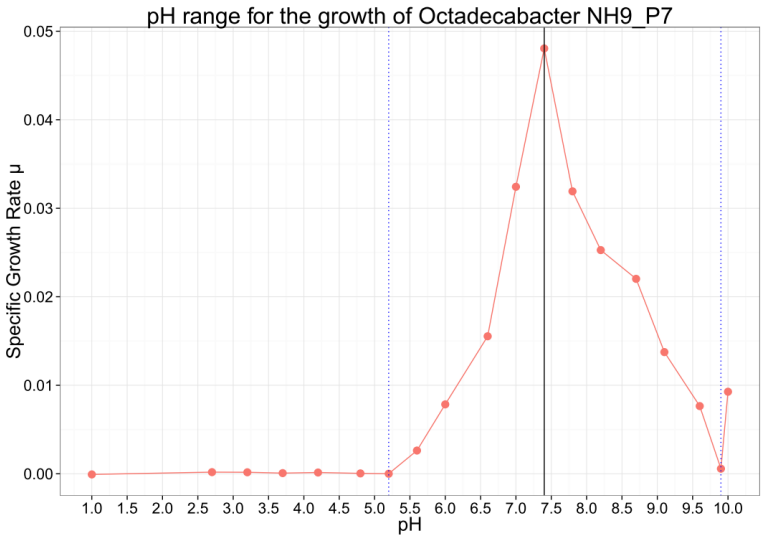

Figure 2. pH range for growth of *Octadecabacter* NH9_P7

According to these results, the strain is able to grow from pH 5.5 to 9.5 with an optimum pH of 7.4.

The temperature range and optimum temperature for growth were determined at pH 7.4 using the same strategy. The optimum temperature for *Octadecabacter* NH9_P7 is 30°C (Figure 3) and it can grow from 4 (data not shown) to 40°C.

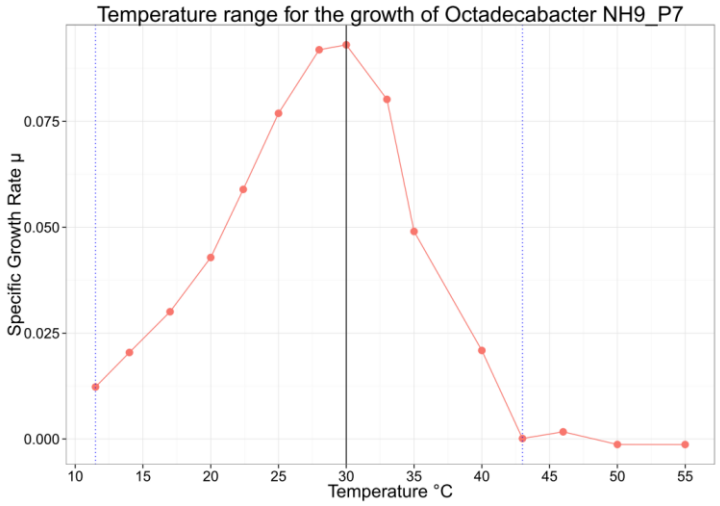

Figure 3. Temperature range for growth of *Octadecabacter* NH9_P7

To determine the optimum NaCl concentration, the strain was inoculated with different NaCl concentrations at pH 7.4 and incubated at 30°C (Figure 4).

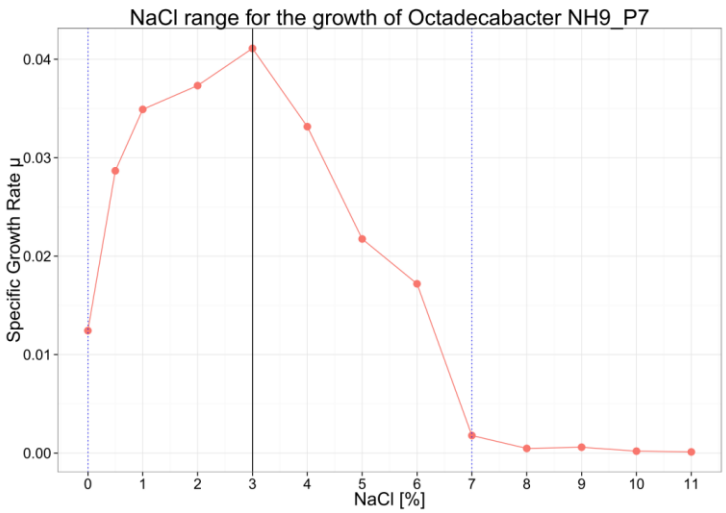

Figure 4. NaCl range for growth of *Octadecabacter* NH9_P7

The *Octadecabacter* strain NH9_P7 can grow with a NaCl concentration between 0 and 6% with an optimum at 3%.

Using the optimum pH, temperature and NaCl conditions, a generation time of 5h was obtained for *Octadecabacter* NH9_P7.

The strain was also tested for utilization of several carbon substrates as well as for the common enzymatic activities (API 20NE and API ZYM tests). For the substrate utilization, the strain was inoculated with artificial sea water amended with 115 different substrates. The growth was monitored by measuring the optical density at 600nm at 24, 48 and 72h. The strain is able to metabolize 30 different substrates (Table 1).

Table 3. List of the substrates metabolized by *Octadecabacter* NH9_P7

[1] Glucose	D-(+)-Arabinose	Cellobiose	Mannose	Maltose
[6] Sucrose	Trehalose	Arabitol	Mannitol	Sorbitol
[11] Casaminos\xe4uren	Caseinhydrolysat	Hefeextrakt	Propionat	Laktat, Milchs\xe4ure
[16] Glycerin	Pepton	N-Acetylglucosamin	Na-Pyruvat	Alanin
[21] Arginin	Ornithin	Prolin	Acetat	Serin
[26] Succinat	Asparat	Glutamat	Fermentierter_Pansenextrakt	Fumarat

The antibiotic resistance of *Octadecabacter* NH9_P7 was also investigated. The results are currently under analysis. All these results, together with the analysis of the draft genome of *Octadecabacter* NH9_P7, permitted to significantly improve its cultivation. A characterization paper is in preparation.

In parallel, we isolated 7 new *Arcobacter* strains from the Channel Sea. These strains are currently under characterization for improving their pH, temperature and NaCl conditions. They will be also tested for several carbon substrates, enzymatic activities and their antibiotic resistance. Three of them were already tested for their optimum pH. The results are currently under analysis. We expect to have all the results in 2 to 3 months. Based on the results, we will then be able to improve the cultivation conditions also for these additional strains.

3- Improved cultivation conditions for new pelagic taxa

CNRS isolated several new genera from surface waters of the Bay of Naples (Mediterranean Sea), the Eilat lagoon (Red Sea) and offshore Roscoff (Western English Channel) (Table 4).

Table 4. New genera of pelagic bacteria isolated from diverse surface waters

Strain	Site of isolation	Closest cultivated species	% similarity (16S rRNA)	Class
NA16D01	Bay of Naples (Med. Sea)	*Thiohalobacter thiocyanaticus* HRh1	92	Gammaproteobacteria
NA17C11	Bay of Naples (Med. Sea)	*Pseudomonas fluorescens* DACG3	94	Gammaproteobacteria
NA18H10	Bay of Naples (Med. Sea)	*Pseudomonas fluorescens* NBRC 14160	94	Gammaproteobacteria
NA19H01	Bay of Naples (Med. Sea)	*Neptunomonas japonica* JAMM 1380	90	Gammaproteobacteria
NA31F03	Bay of Naples (Med. Sea)	*Parvularcula oceani* JLT2013	91	Alphaproteobacteria
NA32H07	Bay of Naples (Med. Sea)	*Marinimicrobium locisalis* ISL-43	92	Gammaproteobacteria
PL154H12	Roscoff (English Channel)	*Pseudomonas fluorescens* KB6	94	Gammaproteobacteria
PL155G12	Roscoff (English Channel)	*Oceanibaculum pacificum*MC2UP-L3	92	Alphaproteobacteria
EIL31D09	Eilat lagoon (Red Sea)	*Hoeflea alexandrii* AM1V30	92	Alphaproteobacteria

These strains were isolated by using low-nutrient heterotrophic media based on the local sea water whose compositions are detailed in Table 5. They include a basal medium we designed and diluted versions of the low-nutrient heterotrophic medium (LNHM) designed by Cho & Giovannoni (2004) and of the "Marine Broth" (Difco) which is based on the medium of Zobell (1941).

Table 5. Composition of the low-nutrient media used for isolation pelagic bacteria.

Basal Medium	Marine Broth 1/1000	LNHM/10
Seawater collected the day of dilution culture (1 L)		
NH_4Cl (10 µM)		
K_2HPO_4 (1 µM)		
Solution of 20 amino-acids (1 µM each)		
Vitamins solution (1ml)		
Trace elements solution (1 ml)		
DMSP (100 nM)		
	1 mg yeast extract 5 mg tryptone	Carbon mixture (1 mg/ each) D-glucose D-ribose Pyruvic acid Glycerol N-acetyl D-glucosamine Ethanol Succinic acid

Most cultures grown in the low nutrient media reached cellular concentrations ranging from 10^4 to 10^6 cells/ml, a situation that prevents their characterization. To improve growth of several new genera, we modified their initial growth medium by increasing concentrations of phosphate and ammonium, amino-acids, and carbon sources of their isolation medium. We also tested diluted versions of the R2A medium, a "low nutrient" medium originally developed by Reasoner *et al*. (1979), to detect coliforms in potable water (Table 6).

Table 6. Optimal media for the growth of newly isolated pelagic bacteria

Strain	Isolation medium	Optimal media for growth	Organic matter (g/l)	Highest cell density (cells/ml)
NA16D01	Marine Broth/1000	Marine Broth/10	0,6	$4.9.10^7$
NA17C11	Marine Broth/1000	R2A/10; RA2/20	0,14-0,28	$2.2.10^7$
NA18H10	Marine Broth/1000	R2A/10; R2A/20	0,14-0,28	$1.7.10^7$
NA19H01	Marine Broth/1000	Marine Broth/10; Marine Broth/20	0,3-0,6	$6.3.10^7$
NA31F03	LNHM/10	Marine Broth/20; Marine Broth/40; Marine Broth/80	0,07-0,3	$3.1.10^7$
NA32H07	LNHM/10	R2A/20	0,28	5.10^4
PL154H12	LNHM/10	Marine Broth/40; Marine Broth/80	0,15-0,3	$3.9.10^5$
PL155G12	LNHM/10	Marine Broth/10; Marine Broth/20	0,3-0,6	$2.6.10^5$
EIL31D09	Marine Broth/1000 + LNHM/10	Marine Broth/10; Marine Broth/20	0,3-0,6	$1.3.10^6$

Our experiments showed that these strains were adapted to low nutrient conditions. Most of them reached cell densities that will allow their characterization. For some however (NA32H07, PL154H12, PL155G12 and EIL31D09), growth conditions that promote higher growth yield are needed.

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Cho, J.C. & S.J. Giovannoni. 2004. Cultivation and growth characteristics of a diverse group of oligotrophic marine Gammaproteobacteria. Appl. Environ. Microbiol. 70: 432-440.

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