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Development of Broad-Range Microbial Minimal Culture Medium for Lanthanide Studies

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Abstract: Rare Earth Elements (REE), also known as Lanthanides (Ln^{3+}), are a group of 17 elements showing peculiar physical and chemical properties. Unlike technological applications, very little is known about the physiological role and toxicity of Ln^{3+} on biological systems, in particular on microorganisms (*e.g.*, bacteria), which represent the most abundant domains on our planet. Up to now, very limited studies have been conducted due to Ln^{3+} precipitation with some anions commonly present in the culture media. Therefore, the development of a minimal medium is essential to allow the study of Ln^{3+}-microbial interactions, limiting considerably the precipitation as insoluble salts. In this regard, a new minimal culture medium capable of solubilizing large amounts of Ln^{3+}, and allowing the growth of different microbial taxa, was successfully developed. Assays have shown that the medium is capable of solubilizing Ln^{3+} up to 100 times more than other common culture media and allowed the growth of 63 bacteria and 5 fungi. The kinetic growth of one yeast and one gram-positive bacterium has been defined, proving to support superior growth and biomass compared to other commonly used minimal media. Moreover, the sensitivity and uptake/absorption of a *Bacillus stratosphericus* strain was tested, highlighting its capability to tolerate concentrations up to 10 mM of either Cerium, Gadolinium or Lanthanum and accumulate different quantities of the three.

Keywords: REE; *Bacillus stratosphericus*; lanthanide toxicity; environmental screening; complexation equilibria; cerium; lanthanide accumulation; *Debaryomyces hansenii*

1. Introduction

Lanthanides (Ln^{3+}), also commonly known as Rare Earth Elements (REE), represent a family of 17 chemical elements, located in the f block of the element periodic table. The versatility and unique physical and chemical properties make them valuable elements for a wide range of applications, contributing to technological and industrial innovations [1]. One of the most peculiar Ln^{3+} characteristics is the ability to emit visible light when excited, producing brilliant and luminescent colors. This property is exploited for production of fluorescent displays, such as those present in liquid crystal televisions (LCDs), flat panel monitors [2] and smartphone [3]. Ln^{3+} are also key components of ferromagnetic materials employed for solid-state memories and production of high-power permanent magnets [4]. Some elements such as europium (Eu) and terbium (Tb), are used in the production of energy in compact fluorescent (CFL) and energy saving lamps [5]; other, as yttrium (Y) and neodymium (Nd), to create active components in lasers [6]. Cerium (Ce), the most widely used Ln^{3+}, is employed as a key component in exhaust automotive catalysts, to reduce harmful emissions [7]. Finally, elements as gadolinium (Gd) are widely used to produce electronic device materials [8].

Despite the widespread use of these elements, the availability of Ln^{3+} is scarce, due to a limited distribution of reserves on Earth, and geopolitical and economic issues that often play a key role in their supply [9]. Furthermore, their extraction is expensive and harmful to the environment, making investments in the search for new mining sites unattractive. [10,11]. About that, many countries are

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trying to diversify their Ln^{3+} supply sources developing new technologies for their recycling and recovering from electronic waste [12,13].

Unlike technological applications, very little is known about the role of Ln^{3+} in biological systems and their physiological or toxic effects on different organisms. Some studies have reported that plants cultivated in the presence of low Ln^{3+} doses improved root development and plant growth [14,15]. On the contrary, the exposure to high doses led to the formation of reactive oxygen species (ROS), biochemical and molecular alterations and growth reduction [14,16]. In studies on aquatic invertebrate (*Sphaerechinus granularis* and *Arbacia lixula)* exposure to Ln^{3+} was associated to nervous or excretory system damage other than to the cytogenetic anomalies [17]. Moreover, in human it was observed a correlation between some diseases (*e.g.*, endomyocardial fibrosis, brain-tumor) and the exposure to La^{3+}, Ce^{3+}, and Gd^{3+} or their accumulation in tissues [18,19]. Regarding microorganisms, more information are available for methylotrophic bacteria, where a biological role was evidenced for Ln^{3+} in the catalysis of certain pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenases (ADHs, MDHs) [20,21]. Other studies have investigated the transport, accumulation and storage of Ln^{3+} in methanotrophs, highlighting the existence of periplasmic proteins with high affinity for Ln^{3+} [22]. Lanmodulin (LanM), a periplasmic protein with a high affinity for Sm^{3+}, Nd^{3+}, and La^{3+}, has been identified in *Methylorubrum extorquens* and it was hypothesized its use in enzymatic functions [23]. Likewise, Lanpepsy (LanP), another periplasmic protein, involved in the lanthanide response, was identified in *Methylobacillus flagellates* [24].

Therefore, the importance and enormous diffusion of Ln^{3+} in human activities poses several problems, including the elucidation of their biological role and effects on the biosphere, the definition of strategies to reduce their environmental impact and the development of technologies for their recovery and recycling.

The aim of our research is to contribute to the improvement of knowledge on Ln^{3+} in the field of microbiology, through the formulation of a suitable non-selective minimal culture medium for the study of a wide range of microorganisms. The use of minimal media is essential for physiological, metabolic, molecular, and genetic studies because it allows the control of experimental conditions. Unlike other culture media, minimal ones do not include complex ingredients in their composition (*e.g.*, peptones, extracts, casamino acids), avoiding or limiting the presence of non-quantifiable and/or unwanted macro-, micro-nutrients and growth factors.

To date, one of the main limitations in Ln^{3+} research is due to poor solubility and consequent precipitation at low concentrations in mineral solution, caused by the reaction with phosphate, sulfate or carbonate ions [25,26]. These precipitates can agglomerate and deposit as pellets, including the microorganisms themselves, interfering with microbial growth and biological activities by different mechanisms, such as: i) reducing the bioavailability of nutrients (i.e. phosphorus, nitrogen); ii) limiting the binding sites involved in the absorption of the latter, by accumulating on the microbial surface and generating a physical barrier [27]; iii) affecting the chemical environment (i.e. pH and ion concentrations) and interfering with the biochemical and metabolic reactions [28]; iv) generating agglomerates which, by adhering to the microbial surface, prevent movement and interactions with other cells or the substrate [29].

For the above reasons, it is essential to formulate a culture medium that minimize the formation of insoluble compounds, reducing their interferences. To avoid the precipitation of Ln^{3+}, the use of organic chelators such as EDTA, EDDS, DTPA, TTHA, is reported in scientific literature [30]. However, it has been demonstrated that many of these compounds are toxic for microorganisms since they interfere with their metabolism [31,32]. Consequently, the addition of a suitable chelator could be a valuable system to overcome this obstacle.

As a contribution to this topic, we report here the formulation of a minimal culture medium, based on citrate buffer and a reduced concentration of phosphate, allowing the solubility of Ln^{3+} to be increased up to a hundred times compared to those obtained in other common minimal media. Citrate acts as a chelator that complexes Ln^{3+}, preventing their precipitation [33]; furthermore, being an organic source of carbon and a growth factor for many microorganisms [34], it is not toxic for most of them.

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2. Materials and Methods

2.1. Preparation and Use of Minimal Culture Medium for Lanthanides

The Minimal Culture Medium for Lanthanides (MCML) was prepared dissolving in 1.0 L of distilled water: 2.0 g NH_4SO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.4 g KH_2PO_4, 10.02 g sodium citrate dihydrate ($Na_3citOH \cdot 2H_2O$; here and in the following the trinegative citrate ion will be indicated as $citOH^{3-}$), 0.94 g citric acid (H_3citOH), 2.0 g D-glucose, 0.0001 g $FeSO_4 \cdot 7H_2O$, 100 µL micronutrient solution (stock solution was prepared dissolving in 100 mL of distilled water: 10.0 mg H_3BO_3, 11.19 mg $MnSO_4 H_2O$, 124.6 mg $ZnSO_4 \cdot 7H_2O$, 78.22 mg $CuSO_4 \cdot 5H_2O$, 10 mg $Na_2MoO_4 \cdot 2H_2O$) and pH was adjusted at 6.0 with few drops of NaOH (2.0 M). The medium was sterilized by autoclaving for 15 min at 120 °C.

2.2. Complexation Equilibria Model

A complexation equilibria model was elaborated in PyES (Python Equilibrium Species) software [35], considering the complex formation between Ce^{3+}, citrate and phosphate at different pH values. For the model elaboration, the total concentration of Ce^{3+} was assumed to be 1.0 mM, while the analytical phosphate and citrate concentrations were the same as reported in MCML (3.0 mM and 40.0 mM, respectively). The complexation constants and reactions considered are described in Table S1.

2.3. Solubility Test

Initially, a Cerium (Ce) solubility test was conducted in MCML using increasing concentrations of $Ce(NO_3)_3 \cdot 6H_2O$ (0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 3.0, 5.0 mM) at different pH (ranging between 2.0 and 8.0). To verify if any precipitate were to form, Ce^{3+} was added to MCML at increasing concentrations (as described above) and then placed in transparent tubes to visualize any precipitates. After 24 hours, in some cases, a white precipitate, at the bottom of the tube, could be observed, highlighting the Ln^{3+} insolubility at the assayed pH and Ce^{3+} concentration.

Afterward, we conducted also other solubility tests either in MCML or in other three commonly used minimal culture media (Table 1): Davis & Mingioli (DM) [36] and Dworkin & Foster (DF) [37], supporting the bacterial growth, and Czapek Dox (CD) [38] for fungi growth. The solubility of three Ln^{3+}: Cerium (Ce), Lanthanum (La), and Gadolinium (Gd), was evaluated as described above by dissolving, separately, increasing concentrations (0.01, 0.05, 0.1, 0.5, 1.0, 2.0 mM) of each Ln^{3+}. In particular, $Ce(NO_3)_3 \cdot 6H_2O$, $La(NO_3)_3 \cdot 6H_2O$, and $Gd(NO_3)_3 \cdot 6H_2O$ were dissolved in: MCML, DM, CD and DF. After 24 hours, a fluffy white precipitate, at the bottom of the tube, highlighted the Ln^{3+} insolubility at the assayed concentrations.

Table 1. Minimal culture medium composition.

Components	Concentration in medium [g L^{-1}]			
	MCML	DM	CD	DF
$(NH_4)_2SO_4$	2.0	1.0	2.0*	2.0
$MgSO_4 \cdot 7H_2O$	0.2	0.1	0.5	0.2
KH_2PO_4	0.4	3.0	-	4.0
K_2HPO_4	-	7.0	1.0	-
Na_2HPO_4	-	-	-	6.0
KCl	-	-	0.5	-
$Na_3citOH \cdot 2H_2O$	10.02	0.5	-	-
H_3citOH	0.94	-	-	2.0
Gluconic acid	-	-	-	2.0
$FeSO_4 \cdot 7H_2O$	0.0001	-	0.01	0.0001

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D-Glucose	2.0	2.0	-	2.0
Sucrose	-	-	30.0	-
L-arginine	-	0.02	-	-
L-tryptophan	-	0.02	-	-
Micronutrients	100 µL**	-	-	100 µL***
pH	6.0	7.0	7.3	6.0

* $NaNO_3$, the nitrogen source typically used in Czapek Dox, was replaced with $(NH_4)_2SO_4$. ** Micronutrients are described in the MCML preparation paragraph. *** DF micronutrients are the same described in MCML.

2.4. MCML Potentiality for the Cultivation of Different Microorganisms

MCML agar plates were prepared to evaluate its capability to allow the cultivation of different microbial species. Solid MCML was prepared adding 15.0 g L^{-1} of agar, and autoclaved for 15 min at 120 °C. The results on the growth were compared with the following agarized (15.0 g L^{-1} agar) DM, CD (either with sucrose 30.0 g L^{-1}, or 2.0 g L^{-1}), DF and with the Plate Count Agar (PCA) rich medium (to 1.0 L of PCA: 5.0 g tryptone, 2.5 g yeast extract, 1.0 g glucose, were added). A reduced amount of sucrose was used in CD to compare the bacterial growth with that obtained in the case of other tested culture media, if the carbon source was glucose (2.0 g L^{-1}). To this purpose, 89 different microorganisms were used. Among these, 82 were bacterial strains of environmental origin (46 Gram-negative and 36 Gram-positive), two were of human health relevance (*Escherichia coli* and the pathogenic *Staphylococcus aureus* [39]), 5 were fungi (2 yeast and 3 molds) (See Table S2). The 89 microorganisms cultivated for 24-36 h on agar plates, were inoculated in 200 µL of Luria-Bertani (LB) liquid medium (to 1.0 L of medium: 10.0 g tryptone, 5.0 g yeast extract, 5.0 g NaCl, were added) in 96-multiwell plate, slowly shaken (60 RPM) overnight at 28 °C for environmental microorganisms, with the exception of the two mesophilic bacterial strains (*E. coli* and *S. aureus*) grown at 37 °C. Then, they were spotted by means of 96-Pin Microplate Replicator on each agar medium and incubated at the corresponding temperatures.

The environmental microorganisms were isolated from both soil and rhizosphere; the two yeasts, *Debaryomyces hansenii* [40] and *Saccaromyces cerevisiae*, were from an in-house microbial collection of Prof. Giovanni Vigliotta (stored at – 80 °C at the Department of Chemistry and Biology of the University of Salerno). Then, *E. coli* (strain JM 109) was purchase at Promega Italia Srl (http: //www.promega.com/products; cat. no P9751), while *S. aureus* was isolated from hospital patients being part of the same collection mentioned above. The microorganism growth was evaluated at one day, two days and five days.

2.5. Determination and Comparison of Growth Parameters

The growth in MCML was compared with that of two minimal media specific for bacteria and fungi, respectively, recognized by world literature and widely used (Table 1). A *Bacillus stratosphericus* strain, previously isolated by Dr. Gianmaria Oliva [41,42], was cultivated in both MCML and DM, whilst *D. hansenii*, in both MCML and CD modified by replacing the glucose with 30.0 g L^{-1} sucrose as carbon source because better used by the yeast [43].

Initially, a colony from fresh culture plates was inoculated in 3.0 mL of LB medium and incubated at 30 °C overnight. Then, the liquid culture was centrifugated at 2,500 RCF for 15 min, the supernatant was discarded, and pellets resuspended in 2.0 mL of sterilize distilled water and vortexed for 30 seconds.

Aliquots of bacterial/fungal suspensions were inoculated in 50.0 mL of the culture media (Table 1) in 250 mL Erlenmeyer flasks, at cellular density of 0.01 measured by optical density at 600 nm (OD_{600}). The flasks were incubated at optimal growth temperatures, 42 °C for *B. stratosphericus* and 30 °C for *D. hansenii*, under constant shaking at 200 RPM. The experiment was conducted in triplicate. The microorganism growth was followed by measuring OD_{600} along time using ONDA UV-20 spectrophotometer (Sinergica soluzioni, Milan, IT). The absence of contamination was repeatedly checked by morphologic analysis (plate assay and microscopy).

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The average growth rate (R) was calculated by determining generation number (n) at the middle of exponential phase, with relation: $[\log(N_2) - \log(N_1)]/\log 2$, where N_1 and N_2 were the number of cells at time t_1 and time t_2 of exponential growth, respectively. R was $n/D_{t\,(t2-t1)}$ and reported as number of generations at hours (h^{-1}) or days (d^{-1}).

The dried biomass was weighed at the end of the growth, in the stationary phase. Specifically, for each culture, a volume of 5.0 mL was withdrawn and centrifugated at 6,500 RCF for 15 min, the supernatant was discarded, and the pellet dried in oven at 70 °C, up to achieve a constant weight.

2.6. Evaluation of Ln^{3+} Toxicity and Accumulation

The toxicity of three different Ln^{3+} (Ce^{3+}, Gd^{3+}, La^{3+}) was evaluated in the case of *B. stratosphericus* strain in agarized MCML, using two very high concentrations: 5.0 or 10.0 mM for $Ce(NO_3)_3{\cdot}6H_2O$; 5.0 or 10.0 mM for $La(NO_3)_3{\cdot}6H_2O$; 10.0 or 20.0 mM for $Gd(NO_3)_3{\cdot}6H_2O$. At first, the salts were dissolved in distilled sterile water and filtered on cellulose acetate membranes (cut-off 0.22 µm), then, the necessary Ln^{3+} amount was slowly added under constant shaking to agarized MCML (at 55 °C) before its polymerization. The *B. stratosphericus* was previously cultivated in liquid MCML overnight at its optimal growth temperatures (42 °C), under constant shaking at 200 RPM, then the cell suspensions were serial diluted with fresh MCML (1:10, 1:100, 1:1000, 1:10000, 1:100000) and 100 µL were spread on MCML agar plates. The latter were incubated for 24 h at 42 °C, and the number (colony forming units, CFU), size and morphology of colonies was determined.

The capability of *B. stratosphericus* to accumulate three Ln^{3+} (Ce^{3+}, La^{3+}, Gd^{3+}) was evaluated by growing the strain in 40 mL of MCML in Erlenmeyer flasks (250 mL) in the presence of 500 µM of each single Ln^{3+} salt. A bacterial sample without Ln^{3+} was considered as control. The flasks were prepared in duplicate and incubated for 24 h at 42 °C under constant shaking at 200 RPM. Afterwards, the cell suspensions were transferred in 50 mL tubes and centrifugated at 10,000 RCF for 15 min. The supernatant was discarded; the pelleted biomass was rinsed three times with distilled water and then dried in oven at 70 °C for two days. Finally, the dried biomass was weighed and mineralized in 1.0 mL of nitric acid (65% w/w) by incubating at 70 °C overnight. The mineralized biomass was diluted in a ratio of 1:30 with ultrapure distilled water to reach a final nitric acid concentration of approximately 2.0%. At the end, the samples were analyzed using an ICP-OES (Optima 7000 DV, Perkin Elmer, Milan, IT) and the three accumulated Ln^{3+} were quantified. Different concentrations from 0.01 up to 10 mg L^{-1} of rare earth element mix for ICP (Sigma-Aldrich, Milan, IT) were used to generate a calibration standard curve ($R^2 = 0.99$).

3. Results

3.1. Solubility in MCML

A minimal culture medium for lanthanides (MCML) was developed by adapting opportune concentrations of mineral macro- and micro-nutrients, citric acid and sodium citrate (0.94 g L^{-1} and 10.02 g L^{-1}, respectively) as citrate buffer. The concentrations of the latter were chosen to limit microbial toxicity and to maintain buffer capability in an optimal pH ranging from 5.5 up to 6.5.

Initially, it was evaluated the MCML capability to avoid, at different pH, precipitation of the Ce, one of the most commonly used Ln^{3+}. Hence, in the MCML, an increase in the solubility of Ce^{3+} was observed at the lower pH values. In fact, the maximum solubility was achieved at pH 2.0 ($[Ce^{3+}] = 5.0$ mM), while at pH above 7.0 it was observed a precipitate already at 1.0 mM Ce^{3+} (Table 2).

Table 2. Solubility of different Ce^{3+} concentrations and pH values in MCML.

	pH						
mM	2.0	3.0	4.0	5.0	6.0	7.0	8.0
0.01	+	+	+	+	+	+	+
0.05	+	+	+	+	+	+	+
0.1	+	+	+	+	+	+	+

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0.5	+	+	+	+	+	+	+
1.0	+	+	+	+	+	+	-
2.0	+	+	+	+	-	-	-
3.0	+	+	+	-	-	-	-
5.0	+	-	-	-	-	-	-

"+" indicates the Ce3+ was still in solution and no precipitation was observed; "-" indicates the presence of a precipitate on the bottom of the test-tube in the form of fluffy white cloud.

3.2. Formation of Ce Complexes

To study the solubility of Ln^{3+} in the MCML, a simplified model was set up that took into account complexation equilibria between Ce^{3+}, phosphate and citrate at different concentrations and pH. The simplest model considered assumes only the hydrolyzed forms of the Ce^{3+} ion. In the 4.0 < pH < 7.0 interval (typically used to cultivate different microorganisms) more than 90.0 % of the ions in solution are in the form of Ce^{3+} (Figure 1). In the second model, it was observed that in presence of 3.0 mM of total phosphate, already at pH 1.35 and 1.0 μM Ce^{3+}, a formation of solid $CePO_4$(s) occurs (Figure S1). In the third model, the complexes formed between Ce^{3+} and citrate are described. In particular, at pH 6.0, the one used for microorganism cultivation in the MCML, the most abundant specie was $Ce(citOH)_2^{3-}$ (~100%, Figure S2). Therefore, it was hypothesized that the addition of citrate to the medium culture as a Ce-complexing agent could prevent the precipitation of solid $CePO_4$(s).

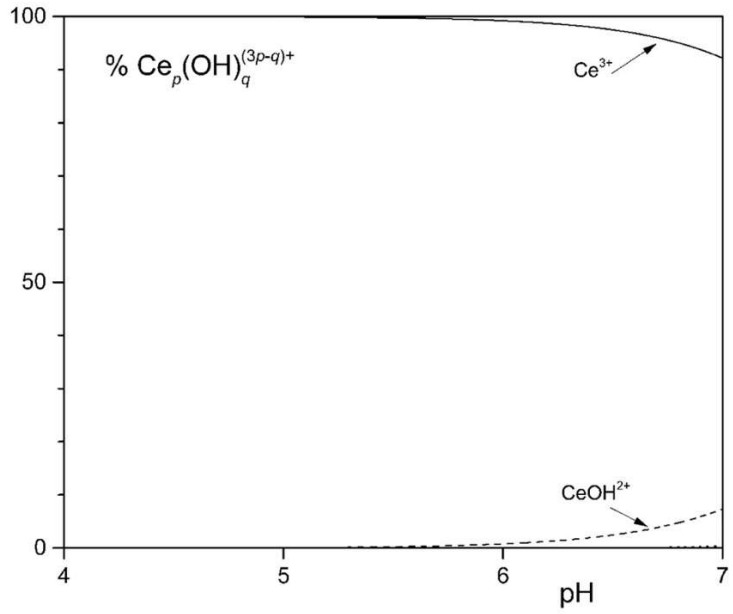

Figure 1. Distribution diagram of the hydrolytic species of the Ce^{3+} ion at 1.0 mM [Ce^{3+}].

The distribution diagram reported in Figure 2 shows Ce-complexes considering in the model 1.0 mM Ce^{3+}, 3.0 mM total phosphate and 40.0 mM total citrate. The abscissa runs up to pH 7.36, when the formation of solid $CePO_4$(s) is observed. Thus, in the data set used to plot the diagram a $logK_s$ of -19 had to be assumed for $CePO_4$(s) freshly formed, instead of the value -26.27 reported for the aged solid, to model the experimental observations showing non precipitation up to pH about 7.5 (Figure 3 – MCML at 0 and 1.0 mM Ce^{3+}).

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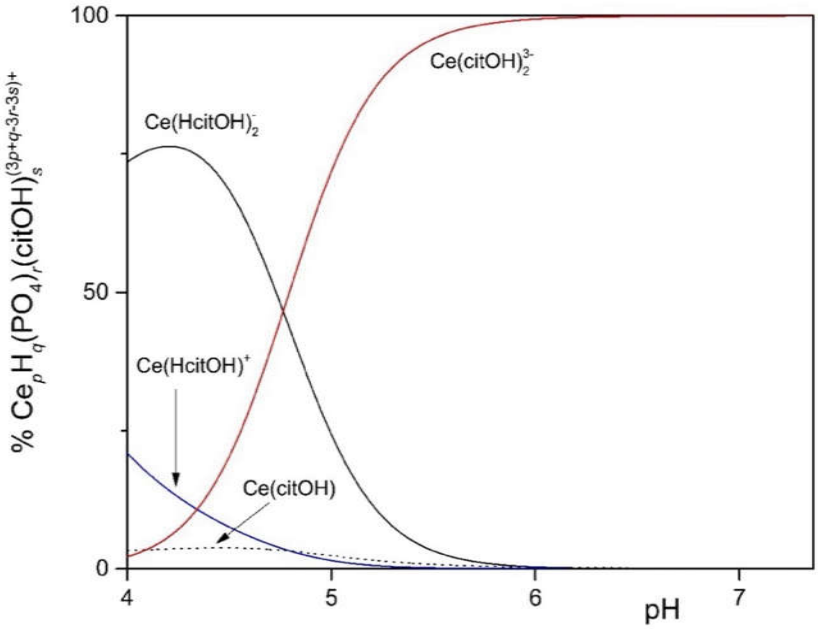

Figure 2. Distribution diagram of 1.0 mM Ce^{3+} in the presence of 3.0 mM total phosphate and 40.0 mM total citrate up to pH 7.36, when the formation of solid $CePO_4$ is observed.

3.3. Solubility of Trivalent Lanthanides in Different Culture Media

In addition to Ce^{3+}, the solubility of other widespread and used Ln^{3+}, such as La^{3+} and Gd^{3+}, was evaluated.

In the MCML, a solubility up to 100 times greater than DF and DM (1.0 mM vs 0.01 mM) was observed for Ce^{3+} and La^{3+}, where a precipitate was already recorded at 0.05 mM (Table 3). While in CD, even at the 0.01 mM Ce^{3+} or La^{3+} concentration, a precipitate, as a white fluffy cloud, was observed (Table 3). Among the three Ln^{3+} in the test tube assay, Gd seems the most soluble one, in fact, in MCML no precipitate was observed up to 5.0 mM, whilst for both DF and DM a solubility no higher than 0.05 mM was recorded. Once again, in CD, even at Gd^{3+} concentration of 0.01 mM, a precipitate was observed (Table 3). Finally, the precipitate was observed at highest Ce^{3+} concentration in the various tested culture media (Figure 3).

Table 3. Maximum solubility (mM) of La^{3+}, Ce^{3+} and Gd^{3+} in different liquid culture media, evaluated at 25 °C and at respective pH values. "-" = Insoluble at minimal tested concentration (0.01mM).

Culture medium	**pH**	**La^{3+}**	**Ce^{3+}**	**Gd^{3+}**
MCML	6.0	1.0	1.0	5.0
Dworkin & Foster	6.0	0.01	0.01	0.05
Davis & Mingioli	7.0	0.05	0.01	0.05
Czapek Dox	7.3	-	-	-

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Culture medium:	MCML	MCML	CD	DF	DM
[Ce(III)] mM:	0	1.0	1.0	1.0	1.0
pH	6.0	6.0	7.3	6.0	7.0

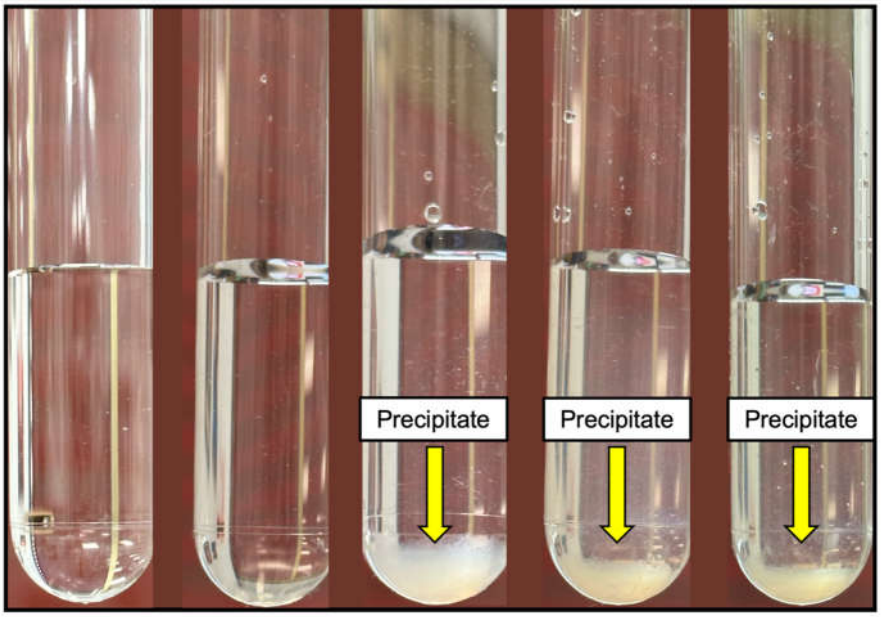

Figure 3. White fluffy cloud precipitate observable in the test-tubes at 1.0 mM of Ce^{3+} in CD, DF and DM culture media.

3.4. Evaluation of MCML Suitability for Microorganism Growth

The potential of MCML on different microorganism growth was evaluated by testing a panel of 84 bacteria (Figure 4, Table S2, Figure S3) and five fungi (two yeasts and three molds) (Figure 5, Table S2). We used the PCA as a control medium, where all microorganisms were able to grow. It was observed that MCML supported the growth of 75 % (63 of 84), whilst DF the growth of 81% (68 of. 84) (Table S2). The DM culture medium allowed the cultivation of only 50% (68 of 84) of the total tested bacteria (Table S2). Finally, the CD medium supported more than 90% of microbial growth for both amount of used sucrose (81 of 84 with 30.0 g L^{-1} sucrose, and 78 of 84 with 2.0 g L^{-1}) (Table S2). Finally, all tested fungi (AL18, DO24, *Sclerotium* sp., *D. hansenii*, and *S. cerevisiae*) grew on all the tested culture media (Figure 5, Table S2).

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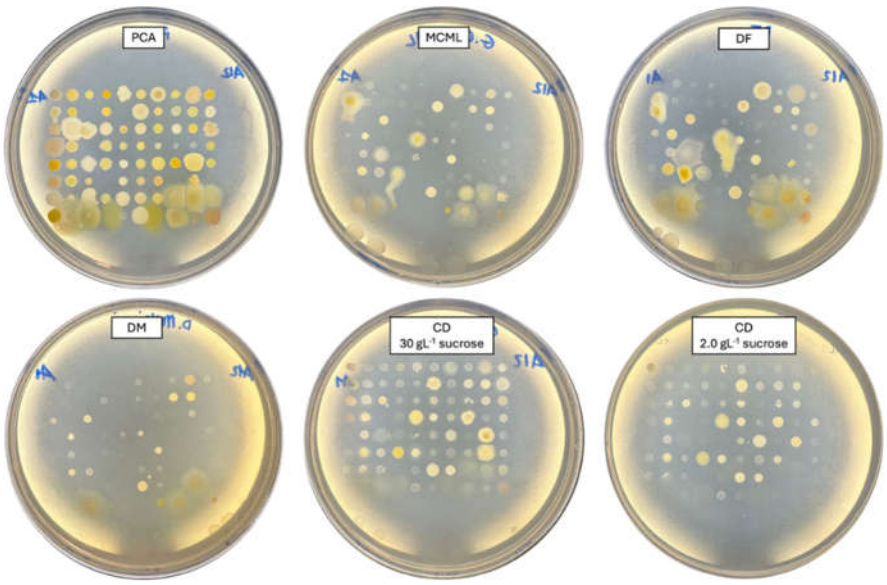

Figure 4. Bacterial cultivation on different agarized culture media after 48 h of incubation.

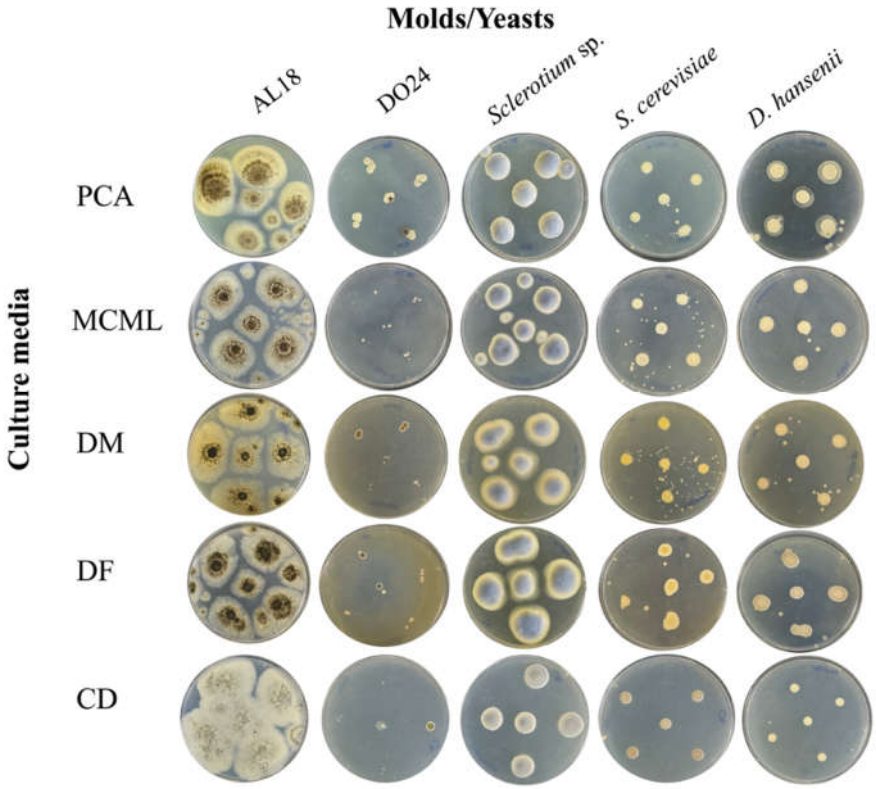

Figure 5. Fungi cultivation and growth on different agarized culture media after 5 days of incubation.

3.5. Evaluation of MCML Medium Growth Parameters

Growth parameters for environmental bacteria and fungi were evaluated in MCML and compared to a reference minimal medium. For this purpose we considered *B. stratosphericus and D. hansenii*, being the most extensively characterized microorganisms among those examined [40–42]. Figure 6 illustrates *B. stratosphericus* baseline growth curves cultivated in two different media: MCML and DM. The growth parameters (cell density at stationary phase, lag phase duration, growth rate) for all baseline experiments were collected and evaluated. It was observed that the lag phase was

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slightly shorter in DM than in MCML medium, < 1.0 h and ≥ 1.0 h, respectively. The average growth rate was greater in MCML (0.7 h^{-1}) compared to DM (0.6 h^{-1}). The final biomass measured in stationary phase was almost twice greater in MCML (0.81 ± 0.01 OD_{600}, this means 5.0 ± 0.2 mg ml^{-1}) compared to DM (0.55 ± 0.07 OD_{600}, 2.5 ± 0.7 mg ml^{-1}).

Likewise, the Figure 7 shows the *D. hansenii* baseline growth curves cultivated in MCML (replacing the carbon source with sucrose 30.0 g L^{-1}) and in the most yeast specific CD; it was observed that the lag phase was < 3.0 hours in CD, while it was > 3.0 hours in MCML. The average growth rate was greater in MCML (0.65 d^{-1}) compared to CD medium (0.49 d^{-1}). Again, approximately two times greater total biomass was obtained in MCML compared to CD medium (1.42 ± 0.05 OD_{600} *vs* 0.80 ± 0.13 OD_{600}; 8.5 ± 0.1 mg ml^{-1} *vs* 4.1 ± 0.7 mg ml^{-1}).

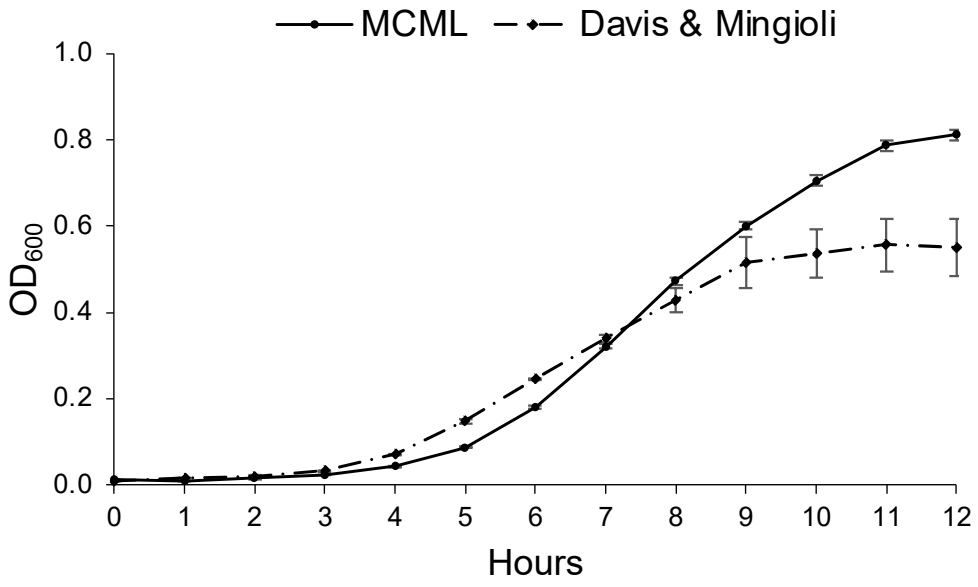

Figure 6. Growth curve of *B. stratosphericus* in MCML and Davis & Mingioli (DM) media.

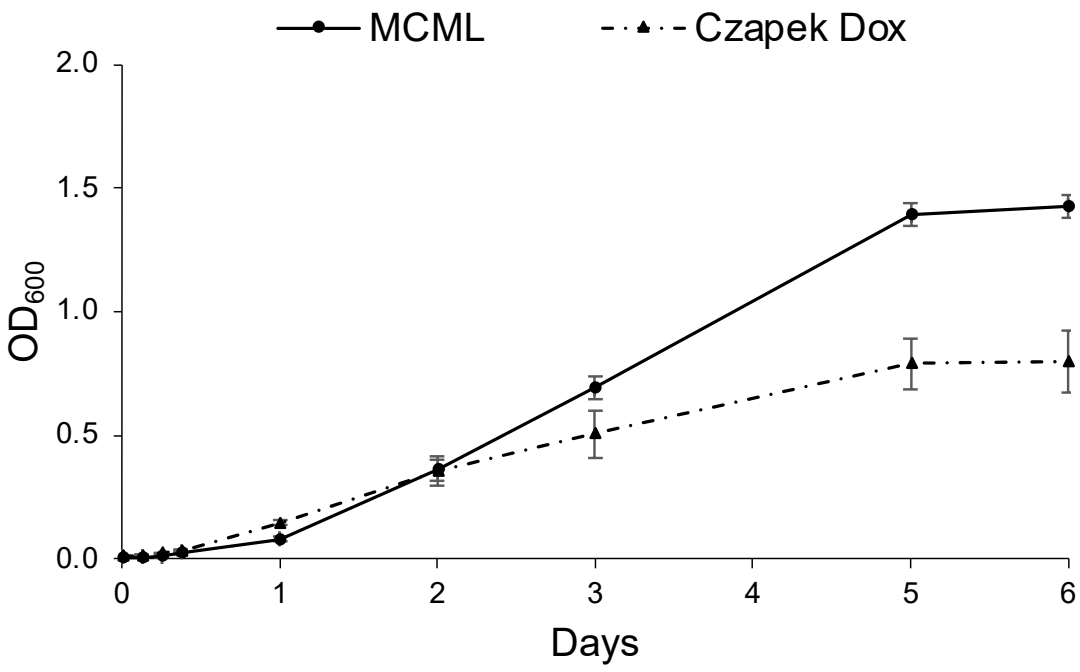

Figure 7. Growth curve of *D. hansenii* in MCML and Czapek Dox (CD) media.

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3.6. Ln^{3+} Toxicity and Accumulation

MCML, due to its ability to solubilize high concentrations of Ln^{3+}, may allow studying different effects of interactions between Ln^{3+} and microorganisms. In this regard, the toxic effect and cellular accumulation of Ce^{3+}, La^{3+}, Gd^{3+} was evaluated for *B. stratosphericus* strain. This gram-positive strain was previously selected for NaCl and heavy metal high tolerance, exopolysaccharides production, and resistance to high temperature (up to 55 °C) [41,42]. The results showed a relation between dose and antimicrobial action for all tested Ln^{3+} as evidenced by a reduction of number and diameter of colonies on agarized medium. At 10.0 mM, Ce^{3+} reduced bacterial population by about 70%, while La^{3+} and Gd^{3+} by 90 and 97 %, respectively (Table 4, Figure S4). Moreover, the colony diameters were reduced of about 77%, 55% and 30%, in the presence of 10 mM Ce^{3+}, La^{3+}, and Gd^{3+}, respectively (Table 5, Figure S4). Finally, the capability of *B. stratosphericus* to accumulate Ln^{3+} was estimated. In particular, the results highlighted a greater accumulation capability for Gd^{3+} (Figure 8 - 7.04 ± 0.88 $\mu g \cdot mg^{-1}$ dry weight), compared to Ce^{3+} and La^{3+} (Figure 8 - 0.69 ± 0.36 $\mu g \cdot mg^{-1}$and 0.076 ± 0.015 $\mu g \cdot mg^{-1}$, respectively).

Table 4. *B. stratosphericus* colony number for each Ln^{3+} treatment at different concentrations (mM). "ND" = not determined; "-" = absence of growth.

Ln^{3+} (mM)	Colony numbers in the follow Ln^{3+} treatments		
	Ce^{3+}	La^{3+}	Gd^{3+}
0 (Control)	$166 \cdot 10^5$	$166 \cdot 10^5$	$166 \cdot 10^5$
5	$121 \cdot 10^5$	$900 \cdot 10^4$	ND
10	$48 \cdot 10^5$	$147 \cdot 10^4$	$54 \cdot 10^4$
20	ND	ND	-

Table 5. Colony diameters (mm) of *B. stratosphericus* measured with ImageJ software, for each Ln^{3+} treatments at different concentrations (mM). Letters a, b, c represents the significant statistical differences in the same treatment at increasing concentration; "ND" = not determined; "-" = absence of growth.

Ln^{3+} (mM)	Colony diameter (mm) in the follow Ln^{3+} treatments		
	Ce^{3+}	La^{3+}	Gd^{3+}
0 (Control)	2.14 ± 0.17 a	2.17 ± 0.47 a	2.05 ± 0.17 a
5	0.89 ± 0.15 b	0.87 ± 0.17 b	ND
10	0.49 ± 0.08 c	0.99 ± 0.12 b	1.43 ± 0.22 b
20	ND	ND	-

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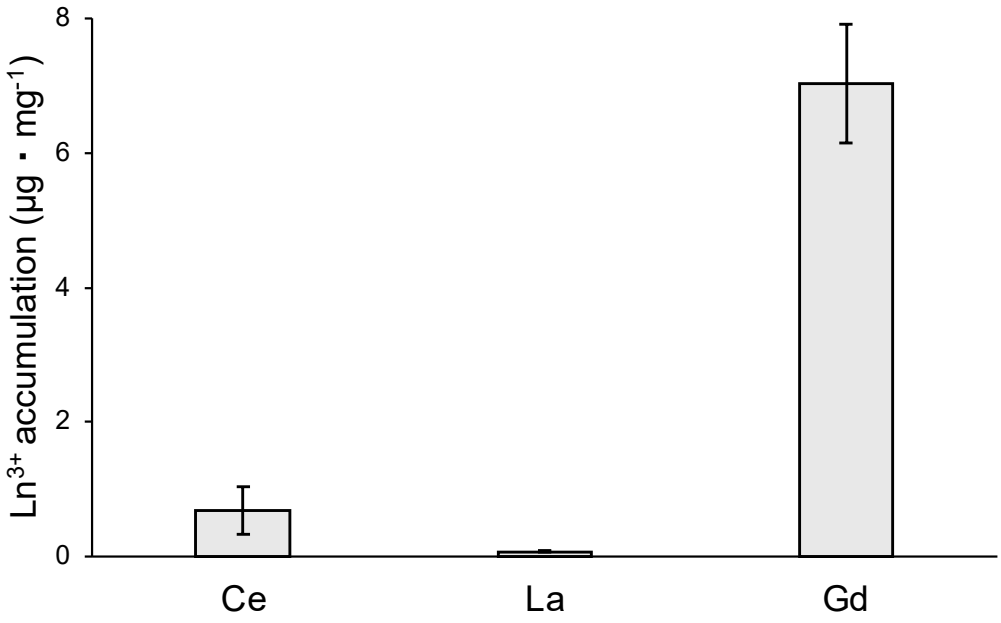

Figure 8. Ln^{3+} accumulation capability of *B. stratosphericus*, expressed as µg · mg^{-1} of dry weight.

4. Discussion

The use of minimal culture media is essential for physiological, metabolic, molecular and genetic studies of microorganisms because it allows control of experimental conditions by reducing the multiple variables. Understanding cellular processes and their regulation are essential for basic and applied research. These research allow to control the growth of microorganisms, or to select and/or engineer strains useful for biotechnological applications such as productive processes (antibiotics, enzymes, drugs and metal bioleaching) or contaminants removal (*e.g.*, inorganics or organics) [44–46].

One of the main problems in studying the interaction between microorganisms and Ln^{3+} is the poor solubility of these elements, and their consequent precipitation in the culture media, even at low concentrations, mainly caused by reaction with phosphate ions. To date, in the scientific literature, only few research studies faced this problem. Rasoulnia et al. (2022) developed a culture medium in which the yeast extract was employed as an alternative phosphate source to reduce precipitation [47]. However, yeast extract due to complex composition, makes the medium similar to a maximal medium and not suitable for minimal media purposes. Groom and Lidstrom (2021) developed a minimal culture medium to study the interaction of Ln^{3+} with microorganism using citrate as a chelator [48]. This medium had a high pH (8.0 – 9.0) and was specific for haloalkaliphilic methanotroph bacteria; moreover, it was tested only with La^{3+}, and up to a concentration of 30 µM, almost 35 times lower than those used in MCML.

Ene et al. (2015) showed the toxicity of Ln^{3+} on *Saccharomyces cerevisiae* using the minimal culture medium "MM" [49]. The authors highlighted the correlation between Ln^{3+} accumulation, both inside and on the cell surface, other than inhibition of Ca^{2+} uptake [50]. However, the Ln^{3+} concentration was not clearly defined, and precipitation problems were only alluded. In other studies, to overcome the precipitation problems, distilled water, water saline solution, or acid conditions, in which these elements are more soluble, were used as analytical medium [51]. This stratagem has been used to evaluate Ln^{3+} absorption by microbial cells as described by Kazak et al. (2021). In this case the absorption was estimated in water saline solutions (0.01 M NaCl), dissolving 1.0 mg·L^{-1} of Ln^{3+}, and it was highlighted that the process is influenced by both pH and bacterial species [52]. However, the use of the above mentioned solutions, where most nutrients are absent or insufficient, can significantly affect cell vitality, altering the metabolism and inducing stress responses, which overall lead to misleading results [53].

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Unlike the above, in our experiment, the developed MCML allowed the solubilization of a large amount of Ln^{3+}, from 1.0 mM (La^{3+}, Ce^{3+}) up to 5.0 mM (Gd^{3+}) at pH of 6.0-7.0, therefore able to support the growth of many microorganisms of different taxa. Moreover, it was observed up to 100 times higher solubility of La^{3+}, Ce^{3+}, Gd^{3+} compared to common tested minimal media (DF, DM, CD).

In our experimental conditions, Ce^{3+} solubility in MCML (Table. 3, Figure 3) didn't agreed with the predicted model obtained assuming a $logK_s$ value of -26.27, as reported in scientific literature for aged solid [data source: https://equilibriumdata.github.io/guide/ (access: January 2024)]. About that, to model the experimental observation, in the data set used to plot the distribution diagram (Figure 2), a $logK_s$ of -19 had to be assumed for $CePO_4$(s) freshly formed, instead of the above reported value for the aged solid. In our model, a solubility product for $CePO_4$(s) about 7 orders of magnitude different from the one reported in the literature is the only way to describe our experimental data. It should be considered that the solubility products, reported in the literature, refer to thermodynamically stable solid phases, which are known to be, by far, more insoluble than the solids formed from supersaturated solutions. In addition, the formation constants available for the Ce(III)-citrate system are questionable, and the existence of complexes not considered in the model is quite probable. Based on these considerations, the model, though very useful, must be considered in some way approximate.

In MCML, the citrate buffer replaces the classic phosphate buffer present in many culture media, in order to reduce the quantity of free phosphate, one of the main causes of Ln^{3+} precipitation in the salt insoluble form [54]. Furthermore, the citrate can complex metals, preventing their precipitation and increasing their solubility [55]. It is also a source of organic carbon and a growth factor for many microorganisms [56,57], although at high concentrations it is toxic [58]. The quantity of citrate solubilized in MCML (~ 11.0 g L^{-1}) is lower than the reported toxicity values (~ 15.0 g L^{-1}) [59], and microorganism growth confirms the tolerability of the concentration employed.

The MCML supports the growth of a high number of environmental microorganisms, both bacteria and fungi. The growth of 82 bacterial strains, belonging to more than 30 different morphological groups, as well as that of five fungi species, were tested. It was observed that MCML supports the growth of 63 bacterial strains (about 75% of tested ones) and 5 fungi compared to a rich medium (PCA), and these results were like those obtained for the other assayed minimal media (DM, CD, DF). It's noteworthy that MCML also supported the growth of *E. coli* and pathogenic *S. aureus* strains.

Among the known genera used in this work, only *Halomonas titanicae* did not grow on MCML. For the Halomonas genus, the citrate concentration used should not be toxic, in fact, Zhang et al. (2020) have observed an excellent growth of *Halomonas* sp. (TDO1 strain) in the presence of citrate up to 30 g L^{-1} [60]. The lack of growth of *H. titanicae* could depend both on the absence of specific nutrients in the culture medium (macro, micronutrients and growth factors) and/or on its physiological characteristics. This bacterial specie is halophilic and requires appropriate concentrations of NaCl [61], therefore, the growth in MCML might require the achievement of suitable osmotic pressure in the medium.

Afterward, the efficacy of MCML was quantified studying the kinetic growth of *B. stratosphericus* and *D. hansenii*, representative of bacterial and fungal taxa, respectively. The growth parameters were compared with those of other common culture media such as DM (for bacteria) or CD (for fungi). The *B. stratosphericus* strain highlighted a lag phase slightly longer in MCML compared to DM, but a better growth rate and final greater biomass production. Similar trend was observed in the case of MCML for *D. hansenii* when compared with CD. This result could be due to the metabolic adaption of both microorganisms at the different medium composition, such as the presence of citrate and a lower phosphate concentration in MCML respect to the reference media. On the other hand, the higher growth rate and amount the biomass produced might be due to the presence of the citrate as a carbon source, in addition to glucose or sucrose.

Finally, we verified the suitability of MCML for Ln^{3+} studies by preliminary evaluation of their biological effects on *B. stratosphericus*. Our data on toxicity indicated a significant dose-dependent antimicrobial action of all compounds, at concentrations of 5-10 mM. Similar results were showed by

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Wakabayashi et al. (2016) for Ce^{3+}, La^{3+}, and Gd^{3+}, highlighting the major toxicity in the case of La^{3+} [62]. However, in their study the toxic effects were determined on the gram-negative *E. coli* and using a rich medium (LB). Moreover, we evidenced the ability *B. stratosphericus* to accumulate Ln^{3+} ions, in particular Gd^{3+}. We didn't investigate the nature of these interaction, absorption to envelope structures or uptake into cells. In this regard, it is know that *B. stratosphericus* strain produces exopolysaccharides (EPSs) [63], which are involved in biofilms formation and in the protection from stress conditions (*e.g.*, contaminated and saline environments). EPSs have anionic groups that bind cations, such as heavy metals, Na^{+}, K^{+}, retaining and accumulating them on the cell surface. This mechanism reduces cellular availability and toxic effects, and similarly, it also could play a crucial role to the accumulation of Ln^{3+}.

5. Conclusions

MCML developed and used in our study could fill the lack in scientific literature of a minimal medium suitable for the cultivation of microorganisms in the presence of great Ln^{3+} concentrations. This also allow an in-depth study of microorganism physiological processes crucial for the development of new relevant biotechnological applications. Furthermore, MCML can be defined as broad-spectrum medium, suitable for the screening of both many environmental microorganisms and of human health relevance. Finally, the results of our study open up new considerations on the need of reliable equilibrium constants relative to the complexes of Ln^{3+} with phosphate, citrate, and other relevant organic ligands typical of the culture media.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, G.V., G.O and S.C.; methodology, G.O. and G.V.; software, G.O and E.V.; validation, G.V., S.C. and E.V.; formal analysis, G.O. and L.D.S.; investigation, G.O. and L.D.S.; resources, G.V. and S.C.; data curation, G.V.; writing—original draft preparation, G.O. and G.V.; writing—review and editing, G.V. and S.C.; supervision, G.V. and S.C.; All authors have read and agreed to the published version of the manuscript." Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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Data Availability Statement: The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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