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Precipitation of Carbonates Crystals by Bacteria Isolated from a Submerged fixed-film Bioreactor used for the Treatment of urban Wastewater

Uad, I.[1], Gonzalez-Lopez, J.[1*] Silva-Castro, G. A.[1], Vílchez, J. I.[1], Gonzalez-Martinez, A.[2], Martin-Ramos, D.[3], Rivadeneyra, A.[4] and Rivadeneyra, M. A.[1]

[1]Departamento de Microbiología, Facultad de Farmacia, Universidad de Granada, Campus Universitario de Cartuja, 18071 Granada, Spain

[2]Departamento de Ingeniería Civil, ETS Ingenieros de Caminos Canales y Puertos, Universidad de Granada, Campus de Fuentenueva, 18071 Granada, Spain

[3]Departamento de Mineralogía y Petrología, Facultad de Ciencias, Universidad de Granada, Campus Universitario de Fuentenueva, 18071 Granada, Spain

[4]Departamento de Electrónica y Tecnología de Computadores, Escuela Técnica Superior de Ingeniería Informatica y de Telecomunicación, Universidad de Granada, Campus Universitario de Almanjayar, 18071 Granada, Spain

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ABSTRACT: The precipitation of calcium carbonate by bacteria isolated from submerged fixed-film bioreactor used for the treatment of urban wastewater in both natural and artificial media was studied. However, carbonate precipitation was detected only when the bacteria grow in media added with calcium. Precipitation took place rapidly, and crystal formation began 3 d after inoculation and the percentage of carbonate-forming bacteria was slightly higher than 90%. Seventeen dominant colonies with carbonate precipitation capacity were the dominant heterotrophic platable bacteria growing aerobically in media added with calcium acetate and calcium chloride. According to their taxonomic affiliations (based on partial sequencing of the 16S-rRNA), the seventeen strains belonged to the following genera of Gram-positive and Gram-negative bacteria: *Artrobacter, Bacillus, Enterococcus, Pseudomonas, Agromyces and Rhodococcus.* All of these strains formed calcium carbonate crystals, precipitated always as calcite crystals which showed large amounts of bacterial fingerprints or bacteria calcification. In addition all the seventeen bacterial strains showed carbonic anhydrase activity. The results of this research suggest that in submerged fixed-film bioreactors used for the treatment of urban wastewater, the precipitation of carbonates through bacterial action could take place *in situ* in the presence of certain concentrations of calcium. Moreover, this biological activity could be useful in the CO_2 and calcium capture in urban wastewater treatments.

Key word: Calcite, Calcium carbonate, Urban wastewater, CO_2 capture, Submerged fixed-film bioreactor

INTRODUCTION

It is widely accepted that microorganisms, contribute to the bioprecipitation of a wide variety of minerals, including carbonates, phosphates, sulphides, oxides, and silicates (Ehrlich and Newman, 2009). Different mechanisms have been proposed for mineral precipitation by microorganisms in natural and artificial habitats (Ehrlich and Newman, 2009; Rivadeneyra *et al.,* 2006a; Rivadeneyra *et al.,* 2006b). Nevertheless, in many cases, the exact role of microbes in this biological process is not known, and there is some controversy as to whether they play a passive or active role, or whether they can directly influence the mineralogy of precipitates (Bosak *et al.,* 2004). In this context, different microbial species have previously been reported and assumed to be associated with biomineral precipitation in diverse environments including bioreactor systems for industrial or urban wastewater treatments (Hammes *et al.,* 2003; Rivadeneyra *et al.,* 2013). Species-specific mineral precipitation has been suggested by several authors (Bosak *et al.,* 2004) though the exact mechanisms of precipitation and the way that this process works within the microbial ecology of the precipitating organism has still not been understood. Several studies have reported the bacterial precipitation of carbonates (Rivadeneyra *et al.,* 2006a; Rivadeneyra *et al.,*2006b ; Sanchez-Roman *et al.,* 2007)

*Corresponding author E-mail:jgl@ugr.es

and have suggested different mechanisms for the bacterially mediated precipitation of carbonate minerals (Rivadeneyra *et al.,* 2010; van Lith *et al.,* 2003). One of the mechanisms most often proposed is the production of ammonium and CO_2 by microorganisms in the presence of calcium and magnesium are the production of calcium or different carbonates of calcium and /or magnesium. Other authors have proposed that the bacteria can serve as a nucleus for mineral precipitation upon adsorbing calcium, magnesium and other metallic cations onto the cell surface, and that the matrix of extracellular polymeric secretions affects mineral precipitation (van Lith *et al.,* 2003).

Because of the amounts of CO_2 in the atmosphere, several authors are actually seeking new strategies to remove carbon dioxide or to prevent their emission. One alternative is to create new equilibrium conditions in calcium and carbon cycles by storing and sequestering CO_2 in the stable form of calcium carbonate (Sharma *et al.,* 2008). As a result, research is now being carried out the use of microorganisms to produce calcium carbonate precipitation that are extremely stable in certain environments. In this context, carbonic anhydrase (CA) is an enzyme that catalyzes the reversible hydration of CO_2 in many organisms. Even though the enzyme has been found in many environments, its precise functions and mechanisms of action are not well known (Albert and Ferry, 1994). CA has an important role in carbonate precipitation in different marine organisms (Liu *et al.,* 2005) and algae (Quiroga and Gonzalez), and in the formation of calcified structures of corals and diatoms (Hopkison *et al.,* 2011). The presence of CA in metabolically diverse species in the Bacteria and Archaea domains is indicative of the significant role if this enzyme in prokaryotic microorganisms (Smith and Ferry, 2000) even though little is known about its function. Sanchez-Moral et al. (2003), suggest that CA could also be responsible for the capture of an important fraction of carbon dioxide by heterotrophic bacteria in underground environments.

Submerged fixed-film bioreactor technologies are wastewater treatment systems in which the organic matter in wastewater provides an energy source for the production of new cells for a mixed population of microbes in an aquatic aerobic or anaerobic environment. In this context, some important genera of heterotrophic bacteria include *Achromobacter, Alcaligenes, Arthrobacter, Pseudomonas and Flavobacterium.* This paper describes a study of the carbonate precipitation by heterotrophic bacteria isolated from a submerged fixed-film bioreactor in culture media made from urban wastewater and mixed liquor amended or unamended with calcium choride, and in artificial culture media (conventional media for crystal precipitation). The objective of this research was to discover which culture conditions influenced biomineral formation caused by the bacteria isolated and confirm the active role played by these bacteria in the mineral precipitation.

MATERIALS & METHODS

The bench-scale plant used for this study (Fig. 1) was based on a design of other bench-scale bioreactor that had been used for urban wastewater treatment in previous studies (Cortes-Lorenzo *et al.,* 2012). It consisted of a methacrylate cylindrical column with a bed size (bioreactor working volume) of 65 cm height and a 15 cm diameter. A porous plastic carrier, Bioflow 9(RVT Process equipment,Inc.), with a surface of 800 m^2/m^3 and a bulk density of 145 kg/m^3 was used as support material(Gonzalez-Martinez *et al.,* 2012). Air was supplied by a diffuser placed on the bottom of the reactor. The bioreactor was fed with influent urban wastewater (72 L/day) coming from the primary settling tank of the municipal wastewater treatment plant (WWTP, "EDAR SUR", EMASAGRA S.A., Granada, Spain). Cleaning cycles of the bioreactor were performed every 15 days in order to avoid filter clogging. The experiments were carried out with an inflow rate of 50 mL/min, HRT (3.8 h) and a constant temperature of 20 °C, according to a previous study (Cortés-Lorenzo *et al.,* 2012). These working conditions had a total duration of 45 days, divided into 3 cycles of 15 days due to the clogging of the biofilter. Influent and effluent wastewater samples from the bioreactor were obtained every 24 h for physico-chemical studies. The Biological Oxygen Demand at 5 days (BOD5), Chemical Oxygen Demand (COD), Total Suspended Solids (TSS), and Volatile Suspended Solids (VSS) were determined according to Standard Methods for the Examination of Waste and Wastewater (APHA, 2005). Physico-chemical data of the bioreactor under experimental conditions were analyzed using the software package STATGRAPHICS 5.0 (STSC, Rockville, MD, USA) to analyze the variance (ANOVA). A significance level of 95% ($p < 0.05$) was selected.

Biofilm samples from the carrier were recovered from the three consecutive cycles of operation (every 15 days) by the following method: 10-100 g of carrier with adhered biofilm were taken and placed in flasks with 50 ml of sterile saline (0.9% NaCl). In order to detach the biofilm from the carrier and disperse cells, the suspensions were sonicated in a bath sonicator (Ultrasonic bath, Selecta) at room temperature for 2 min at 40 kHz (0.05 W/ml) and then placed in an orbital shaker at 155 rpm for 1 h (Vilchez *et al.,*2006). The process was performed twice. The suspensions of

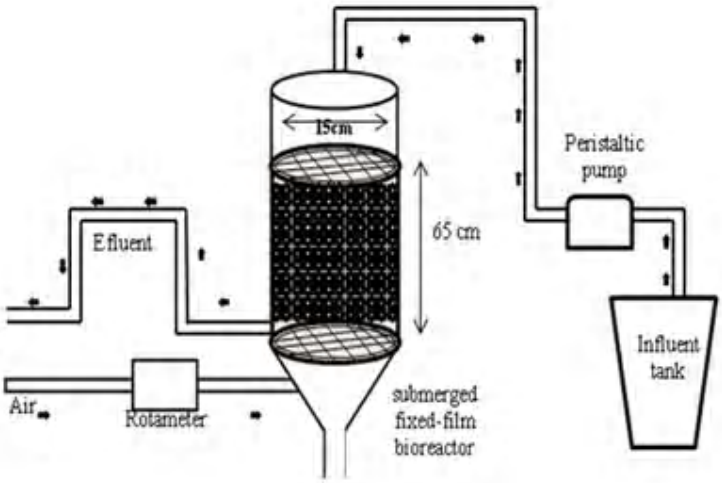

Fig.1. Flow diagram of submerged fixed-film bioreactor constructed at bench scale and used in this study

biofilm material were then used for the isolation of cultivable heterotrophic bacteria.

Aliquots (1 ml) of the biofilm samples were serially diluted and spread on wastewater solid media (WWM), mixed liquor solid media (MLM) and artificial solid media (B4). WWM medium and MLM medium was composed only of urban wastewater and mixed liquor of the submerged fixed-film bioreactor, respectively. The B4 medium (specific medium for carbonate precipitation) was composed of 4.0 g/L yeast extract and 2.5 g/L calcium acetate. To obtain solid media, 18 g/L Bacto-Agar was added, and the pH was adjusted to 7.2 with 0.1 M KOH. Wastewater samples for the preparation of the media were collected from the wastewater treatment plant of the city of Granada. This wastewater was taken from the primary settling tank of the treatment plant. The average concentration of calcium in the wastewater was 15 mg/L. The mixed liquor samples were collected from the bench-scale experimental plant located in the Water Research Institute (University of Granada). The average composition of calcium in the mixed liquor was 12 mg/L.

All the inoculated solid media, after 72 h of aerobically cultivation at 25 ºC showed single, morphologically well-formed colonies. Isolated representatives of the dominant colonies were spread on plates containing WWM, MLM and B4 solid media. The plates were aerobically incubated at 25 ºC for 30 d and checked periodically for the presence or absence of crystals using optical microscopy. Isolated representatives of the dominant colony morphologies with carbonate-forming capacity in B4 medium (seventeen major colony types) were selected and purified, by restreaking them twice. All the experiments were carried in triplicate. For the study of the carbonate precipitation, all the selected isolated (major colony types) were surface-inoculated onto B4 solid medium and incubated aerobically at 25 ºC. Thirty days after inoculation, precipitates were removed by cutting out pieces of the media, which were placed in boiling water to dissolve the agar. The sediments were re-suspended and washed in distilled water to free of impurities. The washed crystals were finally air-dried at 37 ºC. Using this methodology, the morphology of crystals was not altered, as observed by optical microscopy both before and after their recovery. A control consisting of uninoculated culture media and media inoculated with autoclaved cells (dead cells) were included in all experiments.

All the isolates (with crystal-forming capacity) were taxonomically identified by analyzing the partial sequence of the gene encoding 16S rRNA. Primers fD1 and rD1 (Weisburg *et al.,* 1991) were synthesized by Sigma Genosis (UK) and used to amplify nearly the full length of the 16S rRNA gene. Fresh cultured colonies of each strain were lysed by the addition of 20 ul of a mixture of NaOH (0.05 M)-SDS (0.25 %, w/v), which was then boiled for 15 min. The lysates were adjusted to 200 ul with sterile water and centrifuged at 2500*g* for 5 min in a table-top centrifuge. The cleared lysates (4 ul) were used as a template for amplification. PCR was carried out by adding the following to the lysates: 1xPCR Gold buffer (Applied Biosystems, Germany); 1.5 mM $MgCl_2$ (Applied Biosystems, Germany); 200 uM dNTPs (Roche Molecular Biochemicals, Germany); 20 pmol of each primer; and 1 U of Ampli-Tag Gold polymerase (Applied Biosystems, Germany). The final volume of the reaction tubes was adjusted to 50 ul.

Reactions were run in a Perkin Elmer GeneAmp PCR system 2400 (Perkin Elmer, Norwalk, CT, USA). The temperature profile was the one previously described by Vinuesa et al. (1988), except for the extension of the initial denaturation step to 7 min, as required, using the Quiaex II kit (Qiagen, Germany). The nucleotide sequence of the purified bands was determined by the dideoxy chain terminator method, using the ABI-PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Norwalk, CT, USA) and automated sequencer ABI-PRISM 3100 Avant Genetic Analyzer (Applied Biosystems, Germany). The sequenced fragment analyzed corresponded to the first 650 bp of the 16-rRNA gene, comprising hypervariable regions V1, V2 and V3 (Neefs *et al.,* 1990). DNA sequences were analyzed using the biocomputing tools provided on-line by the European Bioinformatics Institute (http://www.ebi.ac.uk). The BLASTn (Altschul et al. 1997) program was used for preliminary sequence similarity analysis, and the ClustalX v.1.8 sofware (Jeanmougin et al. 1998) was used for sequence alignment. Phylogenetic and molecular evolutionary analysis was conducted using MEGA version 4 (Kumar *et al.,* 2001). A *p*-distance based evolutionary tree was inferred using the Neighbour-Joining algorithm. The bootstrap test was conducted to infer the reliability of branch order, with a round of 1000 reassembling. Bootstrap values below 50% are not show in the tree.

Carbonic anhydrase (CA) assay was performed for the seventeen isolated bacteria according to the method previously reported by Ramanan et al. (2009). The assay is based on p-nitrophenol-acetate hidratation to p-nitrophenol and acetate in presence of CA enzyme of p-nitrophenyl-acetate(p-NPA) to p-nitrophenol and acetate that produces a yellow coloration. Bacterial srains were inoculated onto TSA agar medium and incubated at 28 ºC for 24 h over a period of 5 d. The plates were subsequently sprayed with a solution of 10 mM of p-NPA(p-nitrophenylacetate), and the positive colonies showed yellow color zone.

The experiments in liquid media were performed inoculating biofilm suspensions on WWM and MLM amended with 2.8 g/L of calcium acetate or calcium chloride. The pH of the medium was adjusted to 7.2. The main goal of this experiments was to demonstrate if the absence of carbonate precipitation in WWM and MLM media without addition of calcium was a consequence of the low concentration of organic matter or was due to the low concentration of calcium in the culture media. One L Erlenmeyer flasks containing 250 mL of WWM and MLM amended with calcium acetate or calcium chloride were inoculated with 10 mL of biofilm suspensions obtained as above described. The cultures were then aerobically incubated at 25 ºC for 30 d. The evolution of pH and calcium concentration were monitored throughout the experiment after 0, 4, 7, 21 and 30 d. pH was measured with a pHmeter Basic 20 /Crison) and calcium concentration was measured with a Perkin-Elmer 5100ZL atomic absorption spectrophotometer with flame photometry, graphite camera and automatic analyzer equipment. Precipitates were removed by centrifugation of the media and the sediments were re-suspended and washed in distilled water to free of impurities. The washed carbonate crystals were finally air-dried at 37 ºC. A control consisting of uninoculated culture media and media inoculated with autoclaved cells (dead cells) were included in all experiments.

The minerals obtained from solid and liquid media were examined by powder X-ray diffraction (PXRD) using a Philips PW 1710/00 diffractometer with a graphite monochromator automatic slit, CuKaα radiation, and an on-line connection with a microcomputer. Data were collected for a 0.4 sec integration time in 0.02 ºC 2θ steps at 40 kV and 40 mA in a 2θ interval between 3-80 ºC. Data were processed using the XPPowder program for a qualitative and quantitative determination of the mineral composition (Martin, 2004). Secondary electron micrographs of crystal precipitates were made with gold-coated samples using a Zeiss DMS SEM (LEO Electron Microscopy, Oberkochem, Germany), operated at an acceleration voltage of 20 kV to examine the micromorphology of the crystals. Selected samples were coated with carbon for energy dispersive X-ray (EDX) microanalysis. High-resolution secondary electron images were prepared with a field-emission scanning electron microscopy (FESEM) LEO 1525 under 2-3 kV on carbon-coated samples.

RESULTS & DISCUSSION

In recent years, there have been important advances in submerged fixed-bed biofilm reactor systems for the removal of pollutants, such as organic matter, phosphate, and nitrogen. Thus, in the last 25 years, intensive research in the field of biological wastewater treatment has shown that fixed-biofilm systems are often more efficient for water purification than conventional suspended activated sludge systems (Weber *et al.,* 2007). The microorganism are attached growth on surfaces of carrier materials has some advantages, such as a long sludge retention time, prevention of washout of biomass, and better process stability in terms of withstanding shock loadings or short-term disturbing effects (Sudarno *et al.,* 2011). Moreover, biofilm also protects microorganisms in hostile environments, e.g. antimicrobial agents, UV

light, and other stressors (Simões *et al.,* 2010; Lyon *et al.,* 2008).

Table 1 shows the average values of the COD, BOD_5, TSS, VSS, and pH detected in the bioreactor (influent and effluent) during the experiments. As previously reported (Osorio and Hontoria, 2001), submerged filter systems have been shown to be highly efficient at removing COD and BOD_5 from urban wastewater. In this way, the submerged fixed-film bioreactor used in our study presenting an average 92% reduction of COD concentration in the effluent with respect to the influent. However, must be remarked that the bioreactor was cleaned to avoid the clogging of the biofilter every 15 days (three times during one experiment), and consequently the elimination of organic matter by filtration must also be considered. The generation of an active biofilm from the microbiota normally present in urban wastewater took place rapidly because of the high microbial population and nutrient concentration in these environments. In our case, a stable biofilm (confirmed by an optical microscope) was observed in the bioreactor after 24-48 h under our experimental conditions.

The formation of calcium carbonate in solid media was only detected in artificial media (B4) containing with 4 g/L of calcium acetate. However no formation was observed in WWM and MLM unamended with calcium acetate or calcium chloride, uninoculated control media, or media inoculated with a high concentration of dead bacteria and thus without metabolic activity. The number of viable heterotrophic bacteria (CFU) per ml of biofilm in WWM, MLM and artificial media (B4) was in the range of 5.0×10^5 and 2.0×10^6. The percentage of carbonate-forming bacteria in artificial B4 medium was slightly higher than 90%. Calcium carbonate formation took place rapidly, and crystal precipitation began 3 d after inoculation. After 10 d, the crystals had significantly increased in quantity and were of a large size.Fig. 2 shows a colony with precipitates of carbonates. Seventeen major colonies types that produce large amounts of calcium carbonate were selected for taxonomical identification.

Table 1. Operational conditions in the submerged fixed-film bioreactor at bench scale during the experiments

Analytical Parameter	Influent urban wastewater	Effluent wastewater after treatment	Reduction (%)	LSD
BOD5 (mg O_2/l)	425.65 ± 97.83^{a}	17.24 ± 6.51^{a}	95.95	23.87
COD (mg O_2/l)	980.26 ± 233.05^{a}	78.47 ± 28.64^{a}	92.00	48.12
pH	7.55 ± 0.21^{c}	7.46 ± 0.26^{a}	-	0.12
TSS (mg/l)	622.43 ± 398.95^{b}	19.48 ± 7.61^{ab}	96.87	4.05
VSS (mg/l)	520.48 ± 153.24^{b}	13.10 ± 6.14^{ab}	97.48	3.86

Average values marked with the same letter are not significantly different, according to the least significant difference (LSD) test ($p<0.05$). The working conditions had duration of 45 days.

Fig. 2. Colony with precipitates of calcium carbonate growth in B4 culture medium

The taxonomic affiliation of the selected strains, based on partial sequencing of the 16S-rRNA gene (V1 to V3 hypervariable regions, ca. 650 nt) are show in Table 2. The strains were divided in six different genera of Gram-positive and Gram-negative bacteria. Sequence comparison with databases demonstrated the affiliation of strain LM2 to *Arthrobacter* sp. (92% identity), LM3 to *Bacillus* sp. (98% identity), LM6 to *Pseudomonas* sp. (94% identity), LM7 to *Bacillus* sp. (96% identity), LM8 to *Bacillus megaterium* (100 identity), LM9 *Enterococcus* sp. (97% identity), LM10 to *Bacillus* sp. (97% identity), LM11 to *Bacillus* sp. (98% identity), LM12 to *Bacillus flexus* (99% identity), LM13 to *Bacillus* sp. (99% identity), LM15 to *Enterococcus* sp. (97% identity), LM16 to *Agromyces* sp. (96% identity), LM17 to *Bacillus* sp. (83% identity), LM18 to *Agromyces* sp. (92% identity), LM19 to *Rhodococcus* sp. (95% identity), LM20 to *Rhodococcus* sp. (76% identity) and LM21 to *Bacillus* sp. (96% identity).

The phylogenetic tree (Fig.3) shows that most of the identified populations were evolutively related to the Firmicutes (64.7% of sequences). Actinobacteria accounted for 29.4% of the identified sequences and were related to Actinomycetales. Phylogenetically, Gammaproteobacteria were represented by only one strain. According to our results, Firmicutes were identified as the dominant group of bacteria involved in carbonate precipitation in the submerged fixed-bed biofilm reactor system. Firmicutes are widespread components of bacterial community in biofilm systems (Calderon *et al.*, 2011) where members of the Bacillales are responsible for the processes of hydrolysis and degradation of organic matter during wastewater treatment, and they display an ability to become part

Table 2. Illustrate the genetic identification and. Carbonic anhydrase activity of 17 selected strains

Strains	16S rDNA gene sequence	% identity	Sequence lengh (pb)	Group	CA	
					1day	5days
LM2	HM19172B *Arthrobacter* sp	92	1484	Actinobacteria	+	+
LM3	GU471207 *Bacillus* sp	98	1440	Firmicutes	+	+
LM6	EU727193 *Pseudomonas* sp	94	1532	γ- Proteobacteria	+	+
LM7	EU912461 *Bacillus* sp	96	1453	Firmicutes	+	+
LM8	EU256392 *B. megaterium*	100	815	Firmicutes	+	+
LM9	GU299788 *Enterococcus* sp	97	1473	Firmicutes	+	+
LM10	JF309227 *Bacillus* sp	97	1404	Firmicutes	+	+
LM11	HQ916744 *Bacilluc* sp	98	1409	Firmicutes	+	+
LM12	HQ285925 *B. flexus*	99	1411	Firmicutes	+	+
LM13	HQ916744 *Bacillus* sp	99	1409	Firmicutes	-	+
LM15	GU299788 *Enterococcus* sp	97	1473	Firmicutes	D1	D1
LM16	HQ713375 *Agromyces* sp	96	1326	Actinobacteria	+	+
LM17	FJ555568 *Bacilluc* sp	83	1445	Firmicutes	+	+
LM18	EU734593 *Agromyces* sp	92	856	Actinobacteria	+	+
LM19	FJ752527 *Rhodococcus* sp	95	1520	Actinobacteria	+	+
LM20	AY0095 *Rhodococcus* sp	76	1474	Actinobacteria	D1	D1
LM21	FM992808 *Bacillus* sp	96	1475	Firmicutes	+	+

CA (Carbonic anhydrase activity) **+:** intense yellow coloration (few seconds); **D1:** doubtful: very weak staining and stain only the cell mass (possibility of Intracellular production of CA, in small amounts)

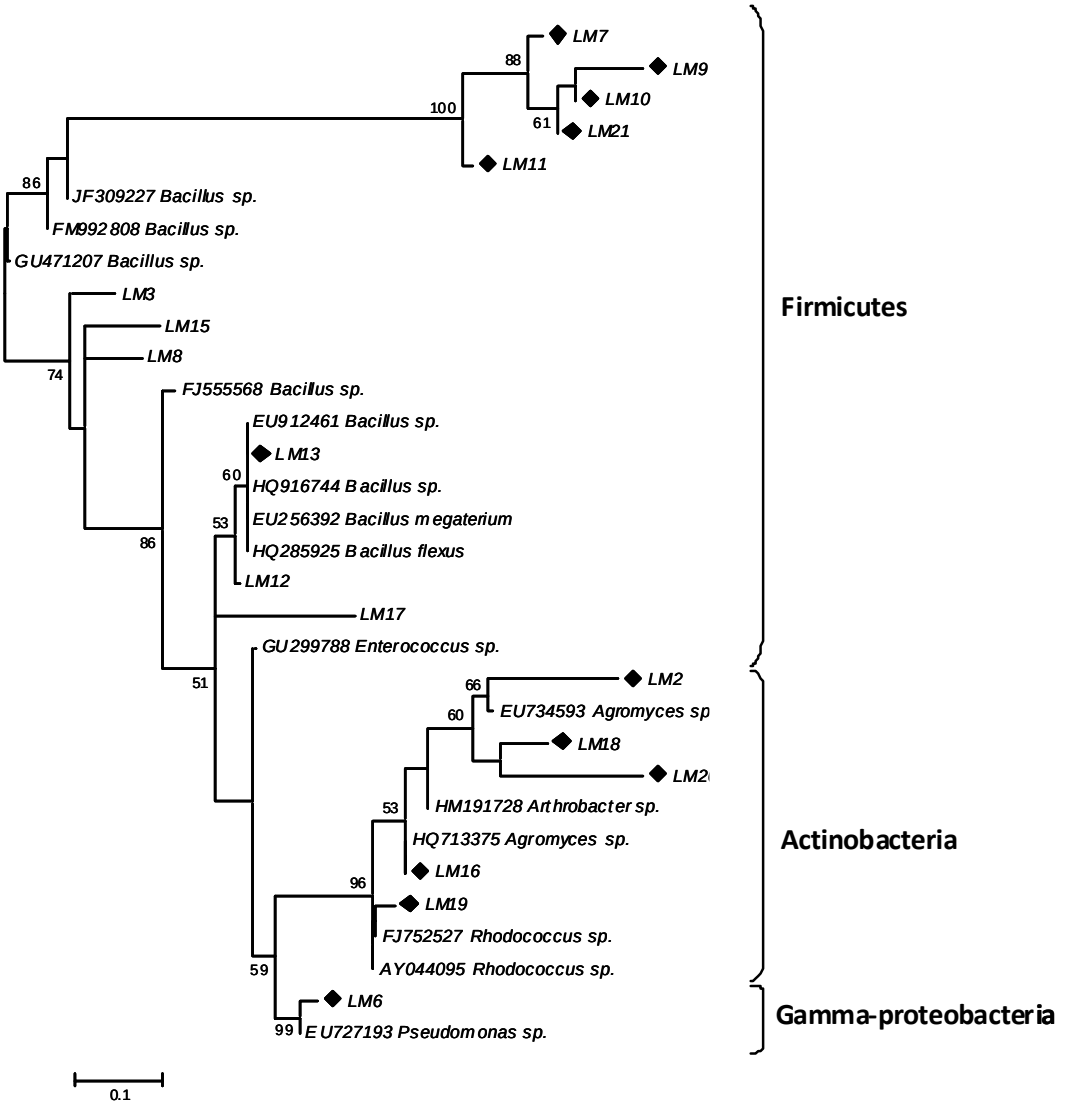

Fig. 3. Phylogenetic relationship based on 16S rDNA sequences of 17 selected strains

of heterogeneous biofims formed under such conditions. Our data also identified Actinobacteria members as prevalent populations in the biofilms with the capacity of precipitate calcium carbonate. Four identified Actinobacteria sequences were phylogenetically close to Actinomycetales (Fig.3). They are commonly present in biofilms and these finding are consistent with the well-known ability of Actinomycetales to colonize solid surface (Alexandrino *et al.*,2001) , where they usually adhere strongly regardless of the surface nature, favored by their production of abundant exopolymers.

Table 2 shows the CA activity of the seventeen strains with capacity to precipitate calcium carbonate, which confirms that CA production is widespread in the bacterial world. Most strains showed after 1 day of growth a strong positive reaction to CA, and became intensely yellow in a few seconds. However the strain LM2 showed a positive reaction after 5 days of growth. This also was observed in the culture media, which indicated the extracellular diffusion of the enzyme. However 2 strains produce a very weak staining only inside the cellular biomass, probably due to the production of small amount of intracellular enzyme. Liu et al. (2005) reported that CA catalyzes the reversible hydration balance from carbon dioxide to bicarbonate in many organisms, decisively influences biological carbonate precipitation in different types of organisms both prokaryotic and eukaryotic. In the same sense, Sanchez-Moral et al. (2003) concluded that CA could also be responsible for sequestration of CO_2 by heterotrophic bacteria. In addition, scientists have

now begun to study the potential use of CA for the storage and capture of CO_2 through its precipitation in the form of insoluble carbonates (Mirjafari *et al*., 2007; Bond *et al*., 2001; Ramanan *et al*., 2009; Sharma *et al*., 2008). In our study, CA activity was detected in all strains with capacity to precipitate calcium carbonate, consequently it might suggested that this enzyme affect the carbonate precipitation. However, more experimental data is needed in order to confirm this preliminary hypothesis, since probably other factor can affect this biomineralization process.

The results of the mineralogical analysis with XRD (Fig. 4, Table 3.) showed that the calcium carbonate precipitated in all the culture media amended with calcium acetate or calcium chloride (solids or liquids) was calcite irrespective of the strain tested. Precipitation of small amounts of amorphous crystals was also detected some media (Table 3). SEM and FESEM observations were also used to study the morphological characteristics of the biominerals precipitated (Figs 5). A variety of shapes were observed. The most important were spheres, hemispheres, and pesudopolyhedral forms, which appeared either in isolation or in group. The most of the crystals formed in all culture media had rough surfaces with small holes, and high porosity (Fig. 5). Many holes were of bacteria-like size and shape, and frequently, mineralized cells were clearly evident. The optical microscopic observation of the plates showed that the majority of the carbonate precipitates were located in the bacterial mass. The EDX analysis (Fig. 5) confirmed the XRD results.

Numerous studies in natural environments have demonstrated the microbial precipitation of calcite (Rivadeneyra *et al.*, 2006a, 2010; Sanchez-Roman *et al.*, 2007). Our study did not detect the formation of minerals such as calcite when bacteria were grown in culture media (liquid or solid) containing urban wastewater as a source of nutrients (WWM medium). In the case of the WWM and MLM, all the bacteria strains assayed grew there very well, and in solid media

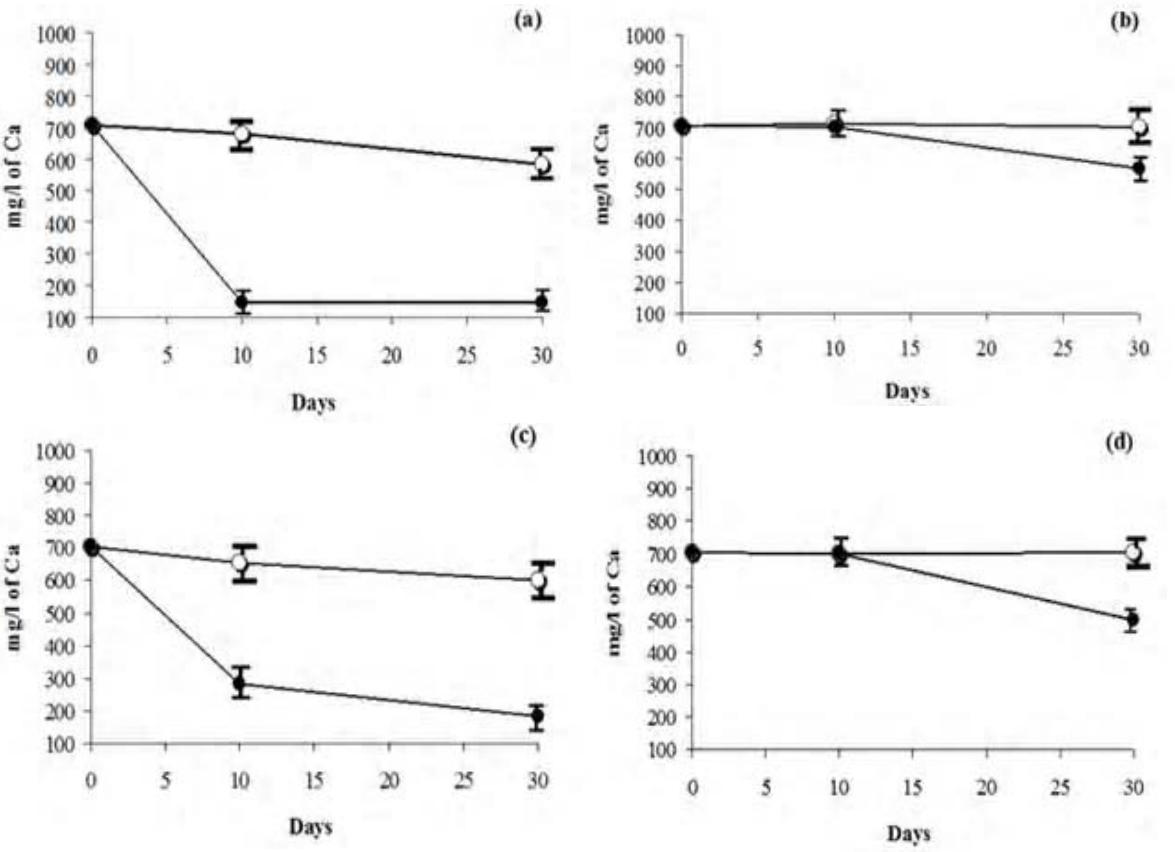

Fig. 4. Evolution of calcium concentration in liquid media. (a) MLM medium amended with calcium acetate, (b) MLM medium amended with calcium chloride, (c) WWM medium amended with calcium acetate and (d) WWM medium amended with calcium chloride. Each experiment included control without addition of calcium. (•) Inoculated medium. (o) Control, uninoculated medium. Values are means ± standard error of three replicates

Table 3. Quantitative analysis (%) of precipitates formed both solid (B4 medium) and liquid mediun (MLM and WWM amended with calcium acetate or calcium chloride, and efficiency of carbonate precipitation in the liquid culture media

Culture medium	Calcite $CaCO_3$	Amorphous	Efficiency
B4	100%	0	-
MLM + $Ca(C_2H_3O_2)_2$	96.5%	3.5%	52.7
MLM + $CaCl_2$	97.4%	2.6%	8.7
WWM + $Ca(C_2H_3O_2)_2$	100%	0	57.9
WWM + $CaCl_2$	100%	0	9.6

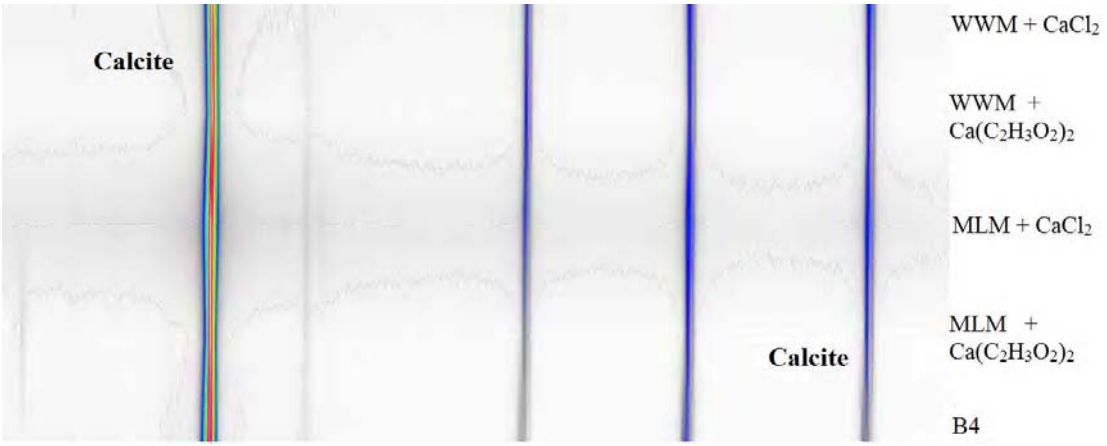

Fig. 5. 2D XRD diagrams of the crystal precipitates in the different culture media. The abscissas show the 2q for CuKa1 radiation. Ordinates show different diffraction patterns. Intensities are in warmer colours, signifying colder colours lower intensities and the warmer colours higher ones. As can be observed, all the samples have a higher crystallinity

formed colonies in 48-72 h though no crystal formation was detected after the 30 d incubation period. However, when bacteria strains was grown in culture media containing calcium chloride or calcium acetate formed calcite after an incubation period of 3 d. Moreover, when the calcium acetate or calcium chloride was added to the urban wastewater an evident precipitation of soluble calcium was detected (Fig. 6). Our data suggest that the calcium concentration in the urban wastewater used in our experiment was not sufficient to produce the precipitation of calcite. In contrast, in culture media amended with high concentrations of calcium, the bacterial populations were able to create the optimal conditions for the formation of calcium carbonate crystals. Probably, the most important finding of this research study is that the heterotrophic bacterial community isolated from a submerged fixed-film bioreactor is able to precipitate soluble calcium as calcite. Our result suggests that in the urban wastewater used in our experiments, the precipitation of calcium carbonate though autochthonous microbiota action cannot take place *in situ*, as a consequence of the low calcium concentration present in this influent. However, if the urban wastewater has a high concentration of inorganic calcium, then biomineralization could be produced.Electron microscopy confirms that the bioliths produced by the bacterial population of the bioreactor often showed calcified bacteria. This verified that calcium carbonate crystals were formed by the accumulation of calcified cells. These results have been previously reported in bioliths formed by certain bacteria isolated in natural habitat (Rivadeneyra *et al.*, 2004, 2006a, 2010). Moreover, FESEM observation (Fig. 4) detected mineralized cells covered with carbonate nanoparticles as previously observed in outer structures of bacteria (Sanchez-Navas *et al.*, 2009; Rivadeneyra *et al.*, 2010). Also, optical microscopic observation of the solid media

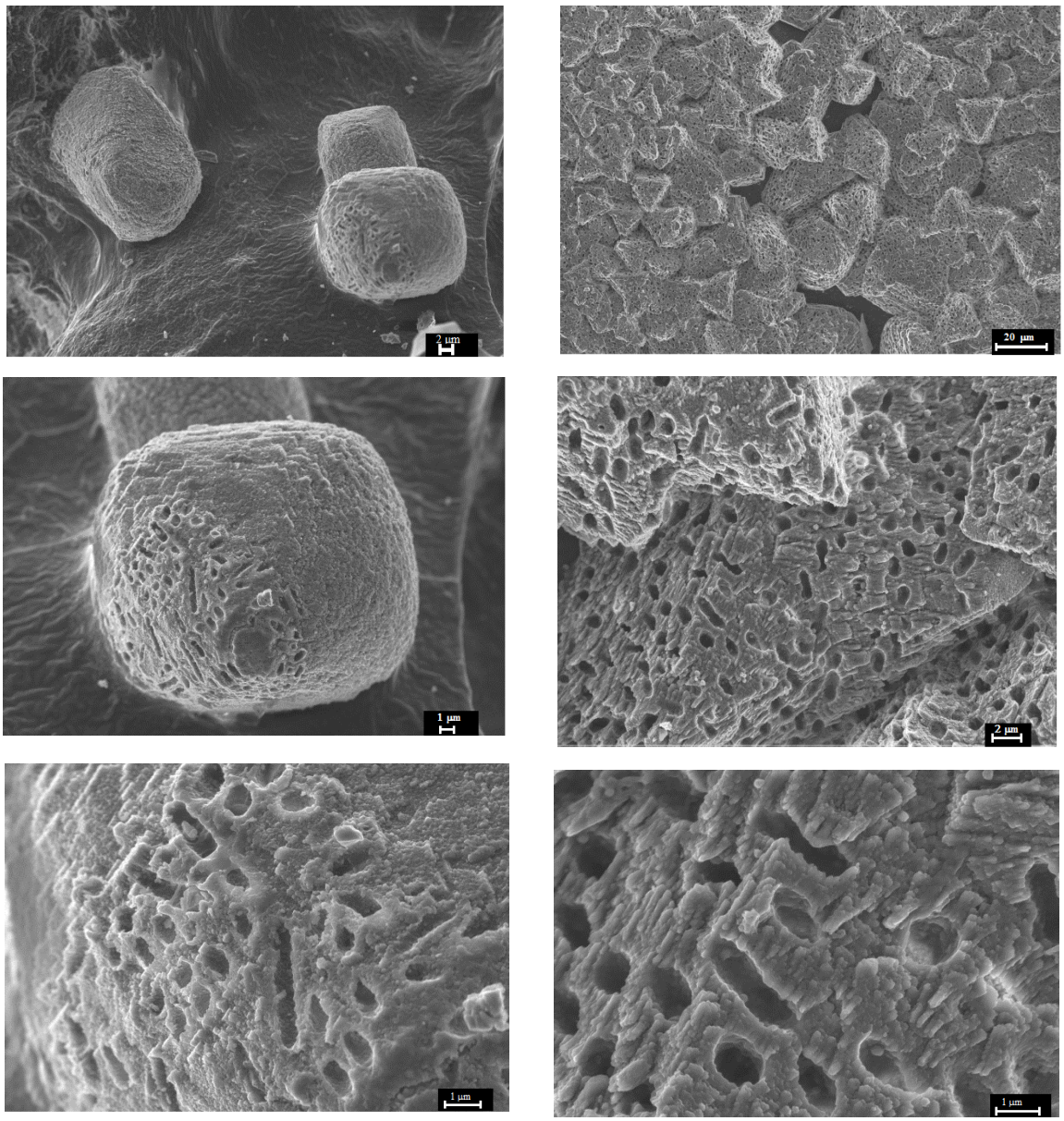

Fig. 6. Scanning electron micrographs (SEM and FESEM) of calcite crystals precipited. 6A: Shows pseudospherulites on mass of bioliths in formation and EDX spectrum at the surface. 6C and 6E: Bacteria fingerprints in the surface; 6B: SEM image of crystal aggregate with pseudopolyhedrical morphology and EDX spectrum at the surface; 6D: Showing a dense aggregate of mineralised bacterial cells; 6F: Note the abundance of calcite nanoparticles delimiting the bacterial cell contours

showed that spherulites were always inside colonies (Fig. 2). Obviously, these results suggest that bacteria induce the precipitation of calcium carbonate and play a role as a precipitation core, although in addition, they modify the environment as a consequence of their metabolic activity as can be seen by the increase in pH in culture media (up to 8.0). Analogues results have been obtained in previous studies by authors such as Dupraz et al. (2004) and Rivadeneyra et al. (2010). Consequently, all the results of this study, which are limited to platable bacteria isolated in laboratory media, verified that heterotrophic bacteria such as *Arthrobacter sp*., *Bacillus sp*., *Pseudomonas sp*., *Enterococcus sp*., *Agromyces sp*. and *Rhodococcus sp*., has an important function in the formation of calcite studied *in vitro*. Moreover, according to this data, could be proposed that the release of CO_2 and NH_4^+ ions (with an increasing pH in the environment) in the

presence of enough concentration of Ca^{2+} ions produces calcium carbonate precipitation. Thus, the metabolization of the organic matter present in the urban wastewater produces CO_2 and NH_4^+ which are totally or partially used in the formation of calcite. The importance of the metabolic activity of the microbiota isolated from the submerged fixed-bed biofilm reactor system is also supported by the fact that no precipitation was detected in the control experiments inoculated with autoclaved cells (dead cells).

CONCLUSION

The results of this study, which is limited to platable bacteria, suggest that bacterial activity has an importance role in the formation of calcium carbonate in submerged fixed-film bioreactors, and that consequently, this is a biomineralization process. The precipitation of calcium carbonate by isolated bacteria from a submerged fixed-film bioreactor used for the treatment of urban wastewater, which contain a high concentration of soluble minerals and metabolizable organic matter, is mainly calcite. However, for the study of biomineralization by heterotrophic bacteria from submerged biofilter system used for urban wastewater treatment, it is advisable to use media derived from urban wastewater amended with high concentration of calcium since environmental conditions present in the bioreactor do not create the optimal circumstances for the precipitation of minerals. Moreover, the results of this research suggest that heterotrophic bacteria populations play an active role in mineral precipitation of soluble calcium from urban wastewater. Finally, could be suggested that the precipitation of calcium carbonate can be useful in the CO_2 capture in same wastewater treatment systems.

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