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Understanding the biochemical characteristics of struvite bio-mineralising microorganisms and their future in nutrient recovery

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Abstract

The biochemical properties of selected microorganisms (*Bacillus pumilus*, *Brevibacterium antiquum*, *Myxococcus xanthus*, *Halobacterium salinarum* and *Idiomarina loihiensis*), known for their ability to produce struvite through biomineralisation, were investigated. All five microorganisms grew at mesophilic temperature ranges (22–34°C), produced urease (except *I. loihiensis*) and used bovine serum albumin as a carbon source. *I. loihiensis* was characterised as a facultative anaerobe able to use O_2 and NO_3 as an electron acceptor. A growth rate of 0.15 1/h was estimated for *I. loihiensis* at pH 8.0 and NaCl 3.5% w/v. The growth rates for the other microorganisms tested were 0.14-0.43 1/h at pH 7–7.3 and NaCl ≤1% w/v. All the microorganisms produced struvite, as identified by morphological and X-ray Powder Diffraction (XRD) analysis, under aerobic conditions. The biological struvite yield was between 1.5–1.7 g/L of media, the ortho-phosphate removal and recovery were 55–76% and 46–54%, respectively, the Mg^{2+} removal and recovery was 92–98% and 83–95%,

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respectively. Large crystals (>300 μm) were observed, with coffin-lid and long-bar shapes being the dominant morphology of biological struvite crystals. The characterisation of the biochemical properties of the studied microorganisms is critical for reactor and process design, as well as operational conditions, to promote phosphorus recovery from waste streams.

Keywords: biomineral formation; struvite; biochemical properties; phosphorus recovery; statistical design

1 Introduction

Biological struvite (bio-struvite) has been identified as a route to recover phosphorus (P) from municipal wastewater streams (Soares et al., 2014). Microorganisms play an important role in struvite bio-mineralisation through different metabolic activities (Sinha et al., 2014) and by precipitation of specific structures or substances for microbial processes (Arias et al., 2017). Five microbial strains, *Halobacterium salinarum, Bacillus pumilus, Brevibacterium antiquum*, *Myxococcus xanthus*, and *Idiomarina loihiensis*, have been reported to be involved in biologically driven struvite formation in liquid streams (Table 1, González-Muñoz et al., 2008; Soares et al., 2014). *M. xanthus*, *I. loihiensis* and *H. salinarum* were reported to produce extracellular polymeric substances (EPS), which may fix cations and contribute to mineral heterogeneous nucleation and precipitation (González-Muñoz et al., 2010, 2008; Merroun et al., 2003). Most of the selected microorganisms can use O_2 as an electron acceptor (Table 1). *H. salinarum* has been reported to be able to use dimethyl sulfoxide

41 (DMSO) as an electron acceptor under anaerobic conditions, and use
42 photophosphorylation in the presence of light (Table 1).

43 *B. pumilus* and *M. xanthus* can use carbohydrates as a carbon source but this does not
44 apply to *B. antiquum* and *H. salinarum* According to the literature, all the selected
45 microorganisms can use protein/amino acids as a carbon source (Robinson, 2014;
46 Trujillo and Goodfellow, 2015). The utilisation of organic carbon sources depended on
47 enzyme production, and the rates of enzyme-catalysed reactions optimally performed
48 under appropriate temperature, pH and salinity ranges (Silva et al., 2016). The selected
49 microbial strains have been reported to grow in pHs from 5.5 to 9, and temperatures
50 ranging from 20–45 °C (Table 1). The halotolerant microorganisms *B. antiquum, H.*
51 *salinarum* and *I. loihiensis* can live in environments containing high NaCl (Gavrish et
52 al., 2004; González-Muñoz et al., 2008; Mesbah and Wiegel, 2005), particularly *H.*
53 *salinarum*, which can survive at extremely high NaCl concentrations (17.4~30.16 %,
54 Table 1).

55 Although some of the biochemical properties and growth conditions of selected
56 microorganism have been reported in the literature, some of the values are controversial
57 and further verification and characterisation is required. Statistical experimental design
58 is recognised as an approach widely used for parameter screening in optimisation
59 studies (Massey et al., 2009). By using such design, Simoes et al. (2017) investigated
60 the significant factors required for *B. antiquum* growth, and maximised the growth rates
61 in wastewater streams by screening and optimising a number of factors.

This study aims to investigate the biochemical properties of the selected microorganisms owing to their capability to produce struvite through bio-mineralisation. For industrial exploitation of microorganisms, the investigation of biochemical characterisation is critical for appropriate processes design and meeting microbial requirements by optimising reactor operational conditions. The temperature, pH, electron acceptor, and organic carbon source are among the most important environmental parameters affecting microbial growth and organic substance synthesis (Silva et al., 2016). Knowledge of such parameters will allow the design of reactors/processes and operational conditions to ensure proliferation of the selected microorganisms, and even out-compete other microorganisms in mixed cultures, for eventual enhanced P recovery by struvite from waste streams.

73 **Table 1 Biochemical properties of the five tested microorganisms**

		B. pumilus	*M. xanthus*	*B. antiquum*	*I. loihiensis*	*H. salinarum*
Strain		MTCC 1640	CECT 422	DSM 21545	MAH1 /CECT 5996	DSM 671
Type		Bacteria	Bacteria	Bacteria	Bacteria	Archaea
Gram reaction		+	-	+	-	-
Cell shape		Rod	Rod	Short rod/ coccoid	Rod	Rod
Size		0.6~0.7 x 2.0~3.0 µm	0.5 x 6 µm	0.6~1 µm	0.3~0.5 x 0.6–2 µm	0.5-1 x 1–6 µm
Motility		+	+	-	+	+
Endospore forming		+	+	-	-	-
O_2 requirement/tolerance		Aerobic	Aerobic	Aerobic	Aerobic	Facultative anaerobic, photophosphorylation at low O_2 concentration with light
Electron acceptor		O_2	O_2	O_2	O_2	O_2, dimethyl sulfoxide
Extracellular polymeric substances synthesis		Not documented	+	Not documented	+	+
Preferred organic carbon source	carbohydrate	Arabinose, mannitol, xylose, glucose, lactose, acetone	Glucose	Not able to directly use	Not documented	Not able to directly use
	protein/amino acid	Casein, lysine,	Amino acids	Casein, amino acid	Amino acid	Lysine, ornithine, arginine
	Other	Citrate, sucrose, D-trehalose, starch, D-glucose, D-arabinose, D-xylose, gelatin	Not documented	Gluconate, urea, gelatin, salicin, sorbitol	$_L$-alaninamide	Gelatin
Growth temperature		20~40 °C, optimum 30~35 °C	14~40°C, optimum 34~36°C	7°C, <37°C; optimum 24~26°C	2 ~ 43°C; optimum 28 ~37 °C	20~55°C, optimum 35~50°C
Growth pH		6~8, optimum at 7	5.5–9.0, optimum at 7	5.5~10, optimum at 7	not documented	5.5~8, optimum at 7
Growth in NaCl		0~2 %	not documented	0~18 %, optimum 3%	0.7~20 %, optimum 2~6 %	17.4~30.16 %, optimum 20.3 %
References		(Robinson, 2014; Shivaji et al., 2006)	(González-Muñoz et al., 2010; Janssen et al., 1977; Merroun et al., 2003; Poza et al., 2004; Robinson, 2014)	(Gavrish et al., 2004; Robinson, 2014; Simoes et al., 2017; Trujillo and Goodfellow, 2015)	(González-Muñoz et al., 2008)	(Losensky et al., 2017; Mesbah and Wiegel, 2005; Mormile et al., 2003; Zinder and Dworkin, 2013)

2 Material and methods

2.1 Microbial strains and culture solution

Five microbial strains were used in this study: *H. salinarum* & *B. antiquum* (DSM 671 & DSM 21545, German Resource Centre for Biological Material, Germany), *B. pumilus* (GB43, LGC Standards, Middlesex, UK), *M. xanthus* & *I. loihiensis* (CECT 422 & MAH1 /CECT 5996, Spanish Type Culture Collection, University of Valencia, Paterna, Spain). The microorganism were grown in synthetic B41 solution comprising 4 g/L yeast extract, 2 g/L $MgSO_4.7H_2O$ and 2 g/L K_2HPO_4 (Da Silva et al., 2000). The solution was autoclaved at 121°C for 20 minutes and cooled to room temperature (20–22°C). For inoculation of each microbial strain; 100 ml synthetic B41 solution in a 250 ml E-flask was inoculated with 0.9% w/v NaCl pre-washed pure cultures of the selected microorganisms that were grown for 96 hours. The E-flasks were sealed with foam stoppers and incubated on an orbital shaker (Stuart model SSL1, Fisher Scientific, UK) at 150 rpm at room temperature. The halophile *I. loihiensis* was grown in B41 solution with 1% w/v NaCl (González-Muñoz et al., 2008).

2.2 Gram staining and enzyme production

Microorganisms, in their early exponential phase of growth (0-8 hours), were Gram-stained using standard methods (Claus, 1992). A KB002™ HiAssorted Biochemical Test Kit (HiMedia Laboratories Pvt. Ltd, India) was used to characterise the pure cultures, according to the manufacturer's instructions. All tests were completed in triplicate and a non-inoculated control was maintained under identical conditions.

2.3 **Statistical design of experiments**

To investigate the impact of growth conditions on microorganisms, a full factorial experiment (FFD) was designed with five factors: temperature, initial pH, NaCl, Ca^{2+}, (by $CaCl_2$) and bovine serum albumin (BSA) as an additional carbon source (Table S1). As factors that are key in optimising the industrial processes involving microbes (Silva et al., 2016), additional variations in NaCl and Ca^{2+} were carried out as they have been inferred to be detrimental to bacterial function and abiotic struvite growth (Le Corre et al., 2005; Rivadeneyra et al., 2006). The tests were based upon low, medium and high levels in relation to characterisation of municipal wastewater and sludge dewatering liquors (Table S1). Temperatures varying from 6–34°C and pH 5.5–8.5 to cover the range of temperatures and pH of municipal wastewater (Tchobanoglous et al., 2003). Ca concentration was adjusted to 28 mg/L (Gassama et al., 2015). The NaCl content varied between 0.5–3.5% w/v, based on characterisation of municipal wastewater and sludge dewatering liquors in different full scale sites in the UK (Simoes et al 2017). (Table S1).Three factors (temperature, NaCl and initial pH) at the 3-level and two factors (Ca^{2+} and BSA) at the 2-level corresponded to $3^3 \times 2^2$ combinations of recipes, which were studied in duplicate and thus generated $3^3 \times 2^2 \times 2 = 216$ tests for each microorganism. The initial and final intact cell counts were examined to generate the overall cell increase that was used as a response to the factors investigated. The experimental data were fitted to a first-order linear regression model or second-order polynomial regression model considering linear and quadratic forms of the independent factors. The response surface methodology (RSM, (Bezerra et al., 2008)) was applied to examine the significant relationship ($p<0.01$) between cell increase and the five growth factors, as

well as the significant two-factor interactions ($p<0.01$). The RSM was also used to determine the optimal conditions that jointly maximise the cell increase by applying a multiple response optimisation. All statistical design and analysis was performed using Minitab 17 (Minitab, 2010).

2.4 **Microbial cultivation under investigated growth conditions**

Microorganisms were grown in 96-well sterile microplates with working volume about 250 µl per well (Corning™, Fisher scientific, UK). Each well contained 234 µl solution and 26 µl inoculum. To prepare the solutions corresponding to the FFD recipes (Table S1), synthetic B41 solution with different NaCl concentrations was autoclaved and mixed with 0.22 µm sterile filtered (Sartorius Stedim Biotech, Germany) BSA and $CaCl_2$ concentrated solutions. The initial pH was adjusted by 0.1 M NaOH and 0.1 M HCl sterile solutions. To minimise liquid evaporation from each well, only the central wells (10 x 6) of the microplate were used for microbial inoculation, and the edge and corner wells of the microplate were used for the non-inoculated controls (Syberg, 2016). Breathable rayon film (VWR Collection, VWR, UK) was used to seal the microplates to stop cross-contamination and to achieve uniform air and gas exchange, while also reducing liquid evaporation for each well. The sealed microplates were then placed inside a cube humidity chamber with four ventilation holes at each bottom corner and with a water reservoir inside. The humidity chamber was kept at constant temperatures of 6, 20 and 34 °C, incubated for 106, 66 and 48 hours, respectively. The application of a humidity chamber was found to reduce liquid evaporation from 150 to 20–25 µl/well by the end of the incubation period.

2.5 **Microbial cultivation at different dissolved oxygen levels**

After investigating microbial growth with the different FFD recipes, the conditions that resulted in the highest increase of intact cell count were repeated but this time ,incubation took at two dissolved oxygen (DO) levels using sacrificial glass vials. For microbial cultivation under aerobic conditions, 30 ml sacrificial glass vials containing 9 ml synthetic B41 solution pre-adjusted in terms of pH, NaCl, Ca^{2+} and BSA were inoculated with 1 ml inoculum and sealed with breathable film. The DO in the 30 mL vials varied between 6–8 mg/L.

For microbial cultivation under anoxic/anaerobic conditions, synthetic B41 solution was pre-adjusted in terms of pH, NaCl and bubbled with $N_{2(g)}$ after passing through a 0.22 μm sterile filter (Sartorius Stedim Biotech, Germany) at a rate of 30 L/min. The 10 ml sacrificial glass vials were sealed with a nontoxic butyl rubber stopper and autoclaved (121°C for 20 min). The DO in the 10 ml vials was close to 0 mg/L. Concentrated solution of $CaCl_2$ and BSA, and 1 ml inoculum were then added using a sterile disposable syringe and needle (VWR, UK). The capability of *I. loihiensis* to grow under anoxic conditions was examined in synthetic solutions with absence of O_2 but with 0.5 g/L $NaNO_3$.

The glass vials were placed inside humidity chambers and incubated on an orbital shaker-incubator (MAXQ5000 M6, Thermo Scientific, UK) at 150 rpm for 120 hours. Samples were taken for examination at regular intervals (4–24 hours) through sacrificial vials. All tests were completed in triplicate, and non-inoculated controls were maintained under identical conditions.

2.6 **Abiotic struvite formation**

Abiotic struvite was prepared by mixing 200 mL 0.05 M $MgSO_4 \cdot 7H_2O$ with 100 ml 0.2 M $NH_4H_2PO_4$, both pre-adjusted to pH 9 with 1 M NaOH (Le Corre et al., 2005). Concentrated BSA solution was added to the mixture at a rate of 4 g/L and incubated on an orbital shaker at 150 rpm at room temperature for 24 hours.

2.7 **Crystal isolation, purification and determination**

The microorganisms were inoculated in 500 mL B41 media in sterile, 1 L Duran bottles, sealed by a breathable film, and incubated under the investigated optimal growth conditions at agitation rate 150 rpm for 120 hours. At the end of the incubation period, the samples were filtered through a 10 μm nylon-mesh filter (Plastok, UK) and the crystals were washed with deionised water twice. The isolated crystals were air-dried at 37°C for 2 hours and weighed to determine crystal yields. The pure crystals were then identified by X-ray powder diffractometer (XRD, D5000, Siemens / Bruker, Germany).

2.8 **Analytical methods**

The intact cell count was examined by the SYBR Green I - propodium iodide co-staining method using a flow cytometer (BD accuri C6, BD Biosciences, US, (Nocker et al., 2017). Solution DO and pH values were determined with a portable DO- meter (HQ40D, HACH, UK) and digital pH-meter (Jenway 3540, Bibby Scientific, UK). The concentrations of soluble chemical oxygen demand (SCOD), PO_4-P, NH_4-N and NO_3-N_2 were monitored with Merck Spectroquant® test kits. Mg^{2+} was measured by atomic absorption spectroscopy (AAS, Analyst 800, PerkinElmer, UK) equipped with flaming

and electrothermal spectrometers. A high-resolution microscope (L-series upright compound microscope, Division of GT vision Ltd, UK) was applied for observation of Gram-stained cultures and crystal morphology in microbial cultures.

3 Results and discussion

3.1 Microbial properties and enzyme production

B. pumilus and *B. antiquum* were identified as Gram-positive and *M. xanthus, H. salinarum* and *I. loihiensis* as Gram-negative, which agrees with previously published information (Table 1). In particular, *B. pumilus* formed crusted two-cell clusters or tetrads in B41 media, which were not observed in the other four microbial cultures. Such cell structures did not grow in size but had the potential to aggregate together or onto the crystal surface. Similar cell structures were observed as mineralised *Thiomargarita* embryo-infesting cells (Bailey et al., 2007), and as silica spheroids onto the cell sheath in microbial silicification (Yee et al., 2003). Thus, the crusted cell structures observed during *B. pumilus* growth in this study is proposed associated with the bio-mineralisation.

It is suggested that *B. pumilus* can form mineral particles along the cell surface during exponential phase in solutions rich in PO_4-P and Mg^{2+}, and the mineral particles firmly attach to the cell surface to form completely encrusted cell minerals. Based on previous work on microbial mineralization occurring at the peptidoglycan wall due to negative charges associated in gram-negative and gram-positive, concentrating cations such as Mg^{2+} (Orange et al., 2009) and the consequential interaction with secreted phosphate

204 group and carboxyl groups which bind into the peptidoglycan framework of gram-
205 positive bacteria (Schultze-Lam et al., 1996).

206 The biochemical characterisation tests demonstrated varied enzyme production amongst
207 the microorganisms investigated. Nevertheless, it was quite remarkable to observe that
208 *H. salinarum*, *B. antiquum, B. pumilus* and *M. xanthus* were capable of using ornithine
209 as a carbon source and produced urease. Urease activity, as well as the degradation of
210 proteins, can generate energy for microbial growth and produce NH_3 as a by-product,
211 which raises the pH and release NH_4-N to combine with PO_4-P and Mg^{2+} for struvite
212 precipitation (Sadowski et al., 2014). Bio-mineralisation of struvite by urease-producing
213 microorganisms in the urinary tract has been reported, leading to the formation of
214 kidney stones, that typically contain 15–20% struvite (Arias et al., 2017; Coe et al.,
215 2005; Prywer and Torzewska, 2010).

216 *I. loihiensis* was the only microorganism investigated in this study that did not produce
217 urease and also the only one that showed a positive reaction of NO_3^- reduction to NO_2^-.
218 The latter is a common phenomenon in anoxic respiration, where NO_3^- was used as an
219 electron acceptor.

220 All five microorganisms showed neither positive nor negative results in terms of lysine
221 utilisation. They were also found to be negative for their ability to use citrate and
222 carbohydrates (including glucose, arabinose, lactose, adonitol and sorbitol) as a carbon
223 source, and for phenylalanine deamination and hydrogen sulfide production. The only
224 exception was that *B. pumilus* showed a 33% positive result for glucose utilisation. The

225 results obtained in this study partially agree with the organic carbon source utilisation
226 presented in Table 1.

227 3.2 **Identification of significant factors to microbial growth**

228 By applying the multi-response surface methodology, each microorganism was grown
229 in optimum conditions (Table 2) within the range of chemical conditions of wastewaters
230 and sludge dewatering liquors (Table S1). BSA was identified to have a significant
231 positive linear correlation ($p<0.01$) with microbial growth. All selected microorganisms
232 were able to use BSA as a carbon source (Table 2). Temperature, pH and NaCl were
233 also identified as being significant for microbial growth for all selected microorganisms.
234 A Ca^{2+} of 28 mg/L was identified to be required for growth of *I. loihiensis* but not for
235 *M. xanthus* growth, and was a non-significant factor for the other three microorganisms
236 (Table 2). In addition, temperature correlated with other factors (carbon source and p,
237 $p<0.01$) within the investigated range of 6-34°C. The growth of *B. pumilus*, *M. xanthus*,
238 and *I. loihiensis* had a positive linear correlation with the temperature and reached a
239 peak value at 34°C, while the relationship between temperature and cell count for *H.*
240 *salinarum* and *B. antiquum* fitted a quadratic trend and the growth peak occurred
241 between 22–24°C. Thus, the optimal growth temperature and enzyme activity for the
242 investigated microorganisms was within the mesophilic range of temperatures (Table 2).
243 Quadratic relationships between pH and microbial growth were also observed. *B.*
244 *pumilus*, *H. salinarum B. antiquum* and *M. xanthus* preferred neutral pH (7.1–7.3),
245 while *I. loihiensis* was observed to adapt to a mild alkaline pH of 8.0 (Table 2).
246 Furthermore, *I. loihiensis*, as a halophile, distinguished itself from the other four
247 microorganisms by its ability to adapt to grow at high NaCl concentration (3.5% w/v),

highlighting its ability to control the increased osmotic pressure due to higher salt concentrations (Robinson, 2014). Whereas the other four microorganisms preferred a reduced NaCl concentration (0.5–1% w/v, Table 2). A coefficient of determination (r^2) was introduced to display the degree that the regression model approximates the real data points, with an r^2 >0.7 typically being considered good (Grace-Martin, 2012). In this study, the coefficient of determination was within the range of 0.71–0.94 (Table 2), and thus the regression model could well explain the divergence of data points from a trend.

Table 2 Significant growth factors (main effect, p<0.01) and preferred growing conditions defined by multi-response surface methodology

	Temperature (°C)	NaCl (% w/v)	pH	BSA (g/L)	Ca^{2+} (mg/L)	r^2
B. pumilus	34	0.5	7.3	4	Ns	0.94
H. salinarum	24	0.5	7.1	4	Ns	0.80
B. antiquum	22	0.5	7.3	4	Ns	0.85
M. xanthus	34	1	7.2	4	0	0.73
I. loihiensis	34	3.5	8.0	4	28	0.71

Ns - Non-significant correlation to microbial growth

a – r^2, *ranging from 0 to 1, indicated the proportion of variation that can be explained by the* regression model. r^2=1 indicates that the regression line perfectly fits the data.

3.3 **Microbial growth at different dissolved oxygen levels**

No lag phase of microbial growth was observed under aerobic conditions (DO = 6-8 mg/L) and the exponential phase occurred within 24/48 hours of incubation starting. The growth rates (μ) for the different microorganisms varied between 0.14 and 0.43

1/hour (Figure 1a). The relatively high growth rate of *B. pumilus* (0.35 1/hour) and *M. xanthus* (0.24 1/hour) under anaerobic conditions distinguished themselves from the other three microbial strains ($\mu \leq 0.04$ 1/hour, Figure 1a). The growth rate of *I. loihiensis* under anoxic condition was 0.12 1/hour (Figure 1a), and >99.5% of NO_3-N was reduced by the end of the incubation time. The final microbial intact cell counts for *B. pumilus*, *B. antiquum*, *M. xanthus*, *H. salinarum*, *I. loihiensis* were 80–94% lower under anaerobic conditions and 66% lower under anoxic conditions, than those under aerobic conditions (Figure 1b). The SCOD removal was 20–27% under aerobic conditions, 0–2.4% under anaerobic conditions. SCOD removal by *I. loihiensis* under anoxic conditions was only *6%* (Figure 1c). Aerobic respiration, using O_2 as an electron acceptor, is known to enable microorganisms to convert energy from carbon sources to adenosine triphosphate production more efficiently than using other electron acceptors (Kader & Saltveit, 2003). Hence, it was unsurprising that higher cell counts and SCOD removal were observed under aerobic conditions (Figure 1b-c). None of the intact cell microbial growth or SCOD removal was observed in non-inoculated controls.

I. loihiensis has been reported to be an aerobic organism (González-Muñoz et al., 2008). However, in this study it was identified as a facultative anaerobe, able to use both O_2 and NO_3 as an electron acceptor. Although *B. pumilus* and *M. xanthus* have been recognised as obligate aerobes (Robinson, 2014), in this study they were found to be facultative anaerobes. There was no report related to *M. xanthus* being a facultative anaerobe, although genome sequencing demonstrated that its common ancestor was a facultative anaerobe (Thomas et al., 2008). Several *B. pumilus* strains have been reported as facultative anaerobes, yet the electron acceptor has not been identified

289 (Alcaraz, 2015). *B*. *antiquum* was observed to be a strict aerobe in this study with a
290 specific growth rate of 0.14 1/hour, agreeing with previously reported growth rates in
291 wastewater with NaCl (3% w/v) and using acetate as the major carbon source
292 (equivalent to 1124 mg chemical oxygen demand/L, (Simoes et al., 2017)). Besides
293 carbon source and electron acceptor, exhaustion of macro/micro-nutrients (Maathuis,
294 2009) or formation of toxic metabolism by-products (Trinh and Srienc, 2009) cannot be
295 excluded as factors affecting the microbial growth.

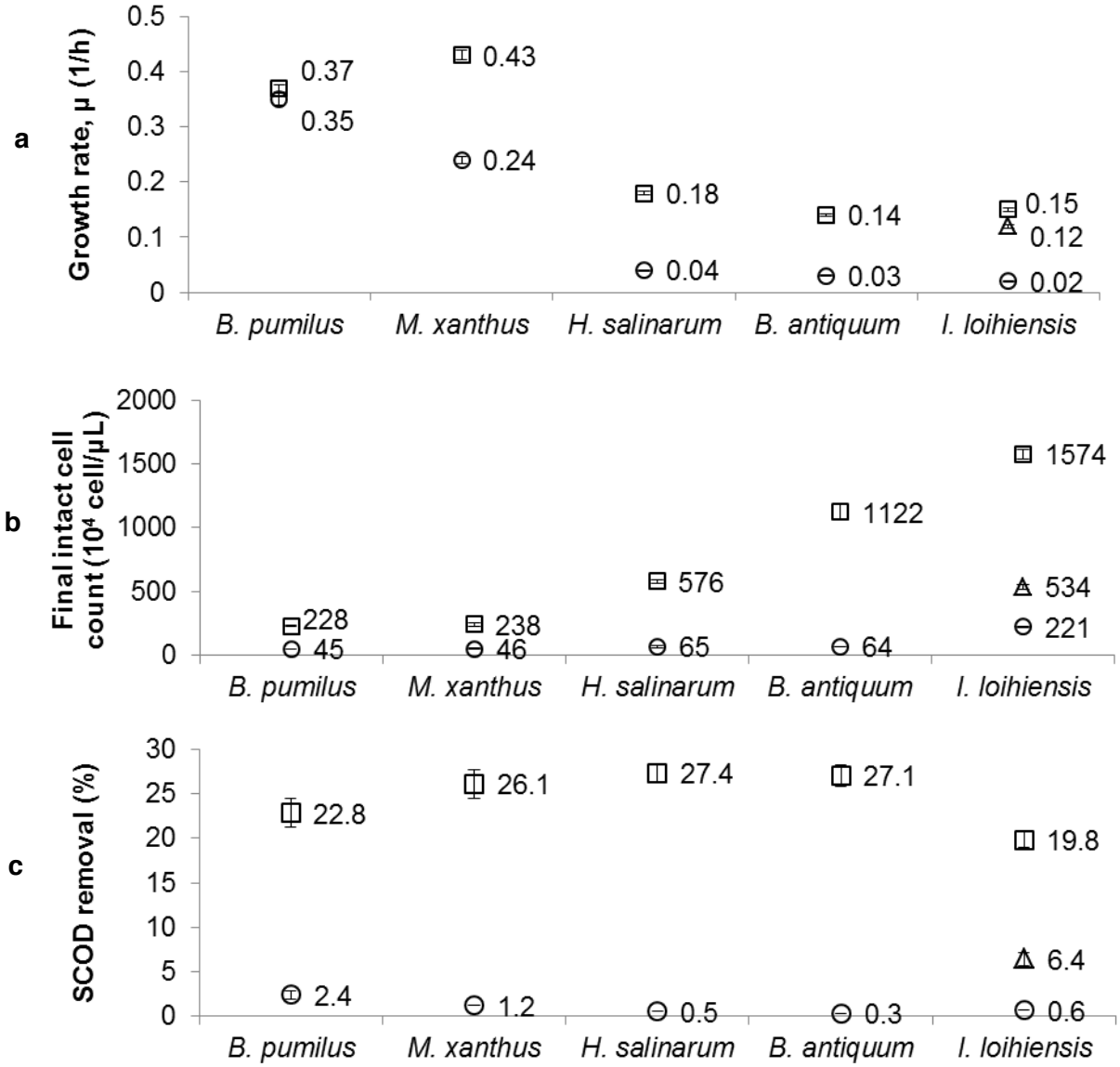

Figure 1 Microbial growth rate (1/h) during exponential growth (0 - 24 /48 h) (a), and intact cell counts (b) and SCOD removal (c). After 120 hour incubation period under aerobic (□), anoxic (Δ) and anaerobic conditions (○). Error bars represent standard deviation obtained from duplicates.

3.4 **Identification of struvite crystals**

All the selected microorganisms produced crystals under aerobic conditions. The XRD diffractions results showed that the curves of isolated purified crystal products met the peak profile of the standard struvite crystals curve (pattern: COD 9007674). The crystals produced by all the microorganisms tested were hence identified as struvite (here called bio-struvite as it was formed through bio-mineralisation mechanism). The bio-struvite and the abiotic struvite crystals presented with the same dominant faces, with miller indices of [011], [111] and [00$\bar{1}$] (Table S2). Besides these three faces, [010] was also found predominant for the bio-struvite produced by *M. xanthus, H. salinarum, B. antiquum*, and *I. loihiensis* (Table S2).

The dominant morphology of the bio-struvite crystals was coffin-lid shape (Figure 2a-b, d-e) and long bar shape (Figure 2c), which have been reported to be among the most typical struvite forms (Tansel et al., 2018). The shape and size of these bio-struvite crystals were different from the relatively small dendritic abiotic struvite (Figure 2h). Abiotic struvite of such dendritic X-shape is typically formed at high pH ≥9 (Ronteltap et al., 2010; Ye et al., 2014). In most microbial cultures grown under aerobic conditions, crystals were observed as early as after 4 hours of incubation (Figure 2g) and these grew larger to more than 300 μm during stationary phase (Figure 2a-b, d-f). In *B. pumilus* culture, a considerable number of crusted tetrad clusters were observed aggregated on the specific bio-struvite crystal surface, particularly the [011] faces (Figure 2g). Bacteria (e.g. *Proteus mirabilis*) was reported to exert control on the bio-struvite crystal morphology (Torzewska et al., 2003). Prywer and Torzewska (2009) proposed a

potential of specific molecular interactions, which related the *P*. *mirabilis* capability of binding to positively charged molecules (e.g. $Mg[H_2O]_6^{2+}$ octahedra) in the crystal surface structure. Such molecular interactions varied with the composition of the microbial secreted biomolecules (e.g. polysaccharide) and its affinity for cations (Prywer and Torzewska, 2009), as well as the charged molecules' type and density on the crystal surface (Sadowski et al., 2014). In this study, the microbial growth may have potential to enhance specific faces of the bio-struvite crystals (e.g. [011], [111], $[00\bar{1}]$ miller indices) and therefore lead to the different crystal morphology (e.g. coffin-lid shape).

The self-assembly of crystals such as contact twinning (Figure 2i-j) and penetration twining (Figure 2k) were observed, along with the parallel grouping of coffin-lid shaped crystals (Figure 2l) and long-bar shaped crystals (Figure 2m). Some bio-struvite crystals were observed with truncated apices, which was related to enhanced [111] end caps (Figure 2n). Similar struvite crystals were observed at low or moderate pH (8–8.5, (Sadowski et al., 2014)).

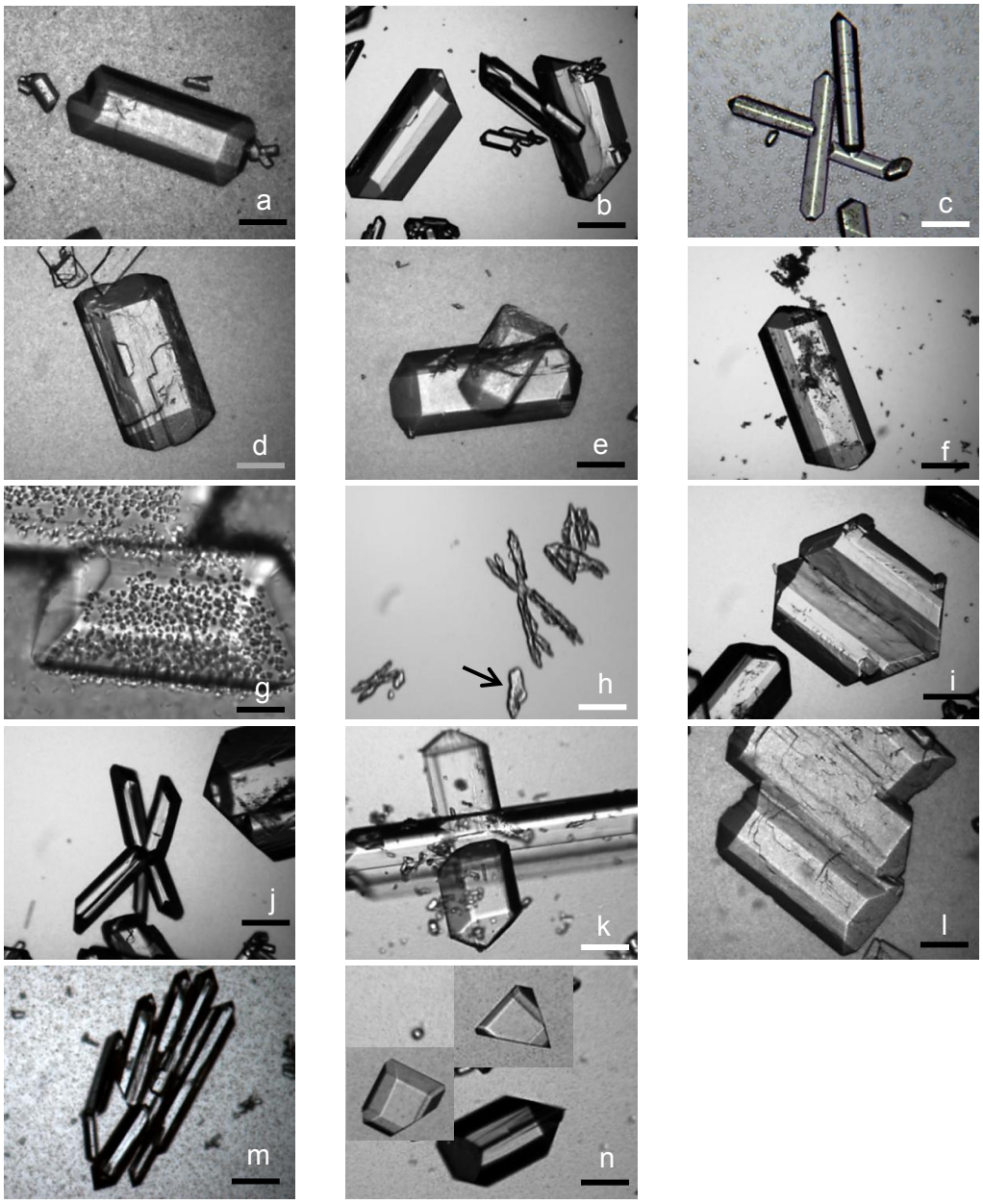

Figure 2 Coffin-lid and long-bar shaped bio-struvite produced in stationary phase by (a) - ***B. pumilus;*** **(b, c) -** ***M. xanthus;*** **(d) -** ***H. salinarum;*** **(e) -** ***B. antiquum*** **and (f) -** ***I. loihiensis*****. (g) Crusted cell cluster aggregated on** ***B. pumilus*** **bio-struvite crystal surface (4 h incubation); (h) - dendritic abiotic struvite crystals, bio-struvite crystals contact twinning (i-k), parallel grouping (l-m), bio-struvite crystals with truncated apices (n), Black bar scale – 88.32 µm, white bar scale – 35.93 µm, grey bar scale – 10.19 µm.**

3.5 **Removal and recovery of ortho-phosphate and magnesium**

The bio-struvite crystal yields under aerobic conditions varied between 1521 and 1746 mg crystals per litre synthetic solution (Table 3). No crystal was collected under oxygen-limiting conditions (Table 3). The removal of PO_4-P and Mg^{2+} by the end of the 120 hour incubation time varied with DO levels. Under aerated conditions, the removal of PO_4-P and Mg^{2+} was between 55–76% and 92–98%, respectively. Under anaerobic conditions, the removal of PO_4-P and Mg^{2+} varied between 1–2% and 2–8% of Mg^{2+}, respectively. Under anoxic conditions, *I. loihiensis* was able to remove 2% of PO_4-P and 32% of Mg^{2+}, from the synthetic media (Table 3).

A mass balance to the nutrients in solution (liquid and crystals <10 μm) demonstrated that considerable amounts of PO_4-P and Mg^{2+} recovered were as bio-struvite (46–54% and 83–95%, respectively (Table 3). Although *B. antiquum* removed a relatively high content of PO_4-P (314 mg/L, 76%) from the synthetic solution, the PO_4-P recovery (48%) by bio-struvite crystals was lower than those for *B. pumilus*, *M. xanthus* and *H. salinarum* (52–54%). Moreover, the Mg^{2+} recovery by *B. antiquum* (84%) and *I. loihiensis* (83%) was observed to be lower than for the other three microorganisms (92–95%).

360 **Table 3 Removal and recovery of PO_4-P and Mg^{2+} at two DO levels by the end of 120 hour**
361 **incubation period.**

	DO (mg/L)	Bio-struvite [a] production (mg bio-struvite/L synthetic solution)	PO_4-P removal	Mg^{2+} removal	PO_4-P recovered by bio-struvite [a]	Mg^{2+} recovered by bio-struvite [a]
B. pumilus	7.2	1700	265 ± 3 mg/L 64%	176 ±1 mg/L 98%	215 mg/L 52%	167 mg/L 93%
	0	0	8 ± 3 mg/L 2%	5 ± 1 mg/L 2%	-	-
M. xanthus	7.2	1746	272 ± 1 mg/L 66%	177 ± 0 mg/L 98%	221 mg/L 54%	171mg/L 95%
	0	0	5 ± 1 mg/L 1%	9 ± 1 mg/L 5%	-	-
H. salinarum	7.8	1692	276 ± 1 mg/L 67%	170 ± 0 mg/L 94%	215 mg/L 52%	166 mg/L 92%
	0	0	7 ± 0 mg/L 2%	7 ± 1 mg/L 4%	-	-
B. antiquum	8.0	1550	314 ± 1 mg/L 76%	173 ± 0 mg/L 96%	196 mg/L 48%	152 mg/L 84%
	0	0	7 ± 3 mg/L 1%	10 ±1 mg/L 6%	-	-
I. loihiensis	6.2	1521	229 ± 1 mg/L 55%	166 ±0 mg/L 92%	192 mg/L 46%	149 mg/L 83%
	0	0	4 ± 2 mg/L 1%	14 ±5 mg/L 8%	-	-
	0[b]	0	9 ± 3 mg/L 2%	58 ±1 mg/L 32%	-	-
control	-	0	0	0	-	-

362 a - Bio-struvite crystals >10 μm

363 b - Anoxic condition with 0.5 g/L $NaNO_3$

364 The synthesis of bio-struvite and removal of PO_4-P and of Mg^{2+} have been reported to
365 depend on microbial growth and metabolism pathways (Sinha et al., 2014). The significant
366 difference of PO_4-P removal and bio-struvite crystal yields between aerobic and anaerobic
367 conditions in this study indicates the importance of DO for P removal and bio-struvite
368 production. Furthermore, the capability of the selected microorganisms, particularly *I*.
369 *loihiensis*, to produce bio-struvite and remove PO_4-P in this study might be underestimated
370 due to the NaCl concentration of 3.5% w/v. It was reported that the increased NaCl could
371 increase the solubility of the struvite phase and therefore lead to inhibition of the bio-struvite
372 crystal size (Rivadeneyra et al., 2006). Significant prevention of bio-struvite production was
373 also observed on sludge dewatering liquors with 3% w/v NaCl (Simoes et al., 2017). The
374 molar ratio of the removed PO_4-P to Mg^{2+} by *B*. *antiquum* under aerobic conditions ([PO_4-
375 P]/[Mg^{2+}] =1.4) was relatively higher than the standard stoichiometric ratio [PO_4-P]/[Mg^{2+}]
376 of struvite, indicating that *B*. *antiquum* may absorb considerable amounts of PO_4-P into cells.
377 Such PO_4-P accumulation within *B*. *antiquum* cells was reported to be relative to the
378 formation of intracellular bio-struvite (Smirnov et al., 2005).

379 *M*. *xanthus* displayed a higher recovery rate of bio-struvite, in comparison with the other
380 microbial strains investigated. Although it removes less P than other strains (66% compared
381 to 76% by *B*. *antiquum*) less of the resource was lost inside biomass or as small crystals.
382 Furthermore, *M*. *xanthus* presented high growth rates (0.43 1/h) and competitive SCOD
383 removal among the others tested (Figure 1). On the other side, its final intact cell count was
384 amongst the lowest, indicating it may be more susceptible to changing conditions
385 experienced in a batch reactor.

3.6 **Implication to the wastewater industry**

Similar to most biological processes in conventional wastewater treatment, bio-struvite production will be ideally applied in open, mixed-culture conditions. The microorganisms enrolled in bio-struvite production are required to out-compete others and become the dominant species in a mixed-microbial culture. The investigation of microbial capabilities and growth of the selected microorganisms in this study can help identify the suitable types of streams (e.g. municipal wastewater, urine, addition of seawater to wastewater) for optimal resource recovery. Streamline reactor and process design, with the most appropriate operational conditions regarding temperature, pH, availability of certain nutrients, and concentrations of NaCl, Ca^{2+} and DO (Table 2). By tailoring processes based on these results the chance for the selected bio-struvite-producing bacteria out-competing other microbial communities in wastewaters increases, whilst efficiency controlling the system can reduce energy and additive costs.

The findings of biochemical characterisation in this study (

400 Table 4) can be compared with existing information (Table 1). This study's findings indicate
401 that *B. pumilus*, *M. xanthus*, *B. antiquum* and *H. salinarum* have the potential to grow in urine
402 due to their ability to produce urease and adapt to lower pH (urine pH 5-7), suggesting that
403 these bacterium would be viable options for urine-separated stream treatment and resource
404 recovery. This also has the benefit of reducing the uncertainty of these bacteria being out
405 competed by mixed-cultures in wastewaters, as urine in typically sterile, improving
406 decentralised system's efficiency and reliability. *I. loihiensis* has the ability to grow under
407 anoxic conditions, alkaline pH, high concentrations of NaCl and Ca^{2+} (e.g. seawater) and can
408 possibly be used in selective chemical pressures for competitive growth. *B. pumilus*, *M.*
409 *xanthus* and *I. loihiensis* have the potential to grow in effluents from mesophilic digesters of
410 temperature around 35 °C. Furthermore, specific wastewater streams characterised by high
411 load of protein/amino acids (e.g. dairy processing wastewater) are proposed as preferred
412 wastewater sources to grow the microorganisms. In all scenarios a well-aerated environment
413 was identified as being essential for bio-struvite production, which can be achieved by pre-
414 existing infrastructure in wastewater treatment plants as secondary treatment process are
415 aerobic, with forced or passive aeration (Tchobanoglous et al., 2003).

416 With the increased knowledge in struvite recovery, researchers have also been investigating
417 its suitability as a fertiliser. This study has shown that bio-struvite produces coffin-lid shaped,
418 tabular crystals, whilst abiotic struvite produced was more dendritic crystal morphologies
419 (Figure 2). The abiotic struvite produced conformed to other studies, where pH exceeded 9
420 (Ronteltap et al., 2010; Ye et al., 2014). The crystal morphologies of bio-struvite (Figure 2)
421 have been demonstrated more suitable for direct land application, as the reduced surface area

from more euhedral crystals, improving its soil retention time as a fertiliser (Shaddel et al., 2019.

Table 4 Summary of biochemical properties of investigated microorganisms and comparison with existing literature (based on Table 1)

	New	Agreement
Enzyme production	• *B. pumilus*, *M. xanthus* and *H. salinarum* produce urease	• *B. antiquum* produce urease
Electron acceptor	• *I. loihiensis* – O_2 and NO_3-N (facultative anaerobe) • *B. pumilus* and *M. xanthus* are facultative anaerobes [c]	• All the tested microorganism can use O_2 as electron acceptor [a]
Carbon source	• *I. loihiensis* cannot directly use carbohydrates, but can use proteins • *B. pumilus* and *M. xanthus* cannot directly use carbohydrates [b, c]	• *B. antiquum*, *I. loihiensis* and *H. salinarum* cannot directly use carbohydrates • *B. pumilus*, *M. xanthus*, *H. salinarum* and *B. antiquum* can use amino acids/proteins
Growth temperature	• *H. salinarum* prefer mesophilic temperature (24°C) [c]	• *B. pumilus M. xanthus* and *I. loihiensis* prefer high mesophilic temperature (34°C) • *B. antiquum* prefer mesophilic temperature (22°C)
Growth pH	• *I. loihiensis* can grow within pH 5.5–8.5, and prefer mild alkaline pH 8	• *B. pumilus*, *M. xanthus*, *B. antiquum* and *H. salinarum* prefer neutral pH (7.1–7.3)
Growth NaCl	• *M. xanthus* prefer 1% w/v NaCl • *B. antiquum* and *H. salinarum* prefer 0.5% w/v NaCl [c]	• *I. loihiensis* prefer 3.5% w/v NaCl • *B. pumilus* prefer 0.5 % w/v NaCl
Growth Ca^{2+}	• Positive effect of Ca^{2+} of 28 mg/L on *I. loihiensis* growth • Negative effect of Ca^{2+} of 28 mg/L on *M. xanthus* growth	

a - Microorganisms produced bio-struvite only under aerobic conditions.
b - The *B. pumilus* were 33% positive for glucose utilisation.
c – Different findings from previous studies.

4 Conclusion

- Proteins/amino acids were the preferred organic carbon sources for the five microorganisms investigated.
- *B. pumilus*, *M. xanthus*, *H. salinarum* and *B. antiquum* were able to produce urease.
- *I. loihiensis* was found to be a facultative anaerobe able to use O_2 and NO_3-N as an electron acceptor.
- The preferred temperature for all selected microorganisms was within the mesophilic range (22–34 °C); most microorganisms preferred a neutral pH and NaCl concentrations less than 1% w/v, whereas *I. loihiensis* preferred a mild alkaline pH 8, high NaCl of 3.5% w/v and the presence of Ca^{2+}.
- The selected microorganisms produced bio-struvite crystals under aerobic conditions. The morphology of crystals produced was dominantly coffin-lid and long-bar shapes.
- The bio-struvite production and PO_4-P removal highly depended on the microbial growth and DO level. At the investigated optimal growing conditions, in the presence of DO, the bio-struvite crystal (>10 μm) yield and PO_4-P removal varied between 1,521–1,746 mg/L and 55–76%, respectively.

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Understanding the biochemical characteristics of struvite bio-mineralising microorganisms and their future in nutrient recovery

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