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# Optimized medium culture for *Acidobacteria* subdivision 1 strains

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**One sentence summary:** New medium formulation named PSYL 5 includes sucrose, as a carbon source, and other compounds (such as  $\text{KH}_2\text{PO}_4$  and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) for members of *Acidobacteria* subdivision 1.

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## ABSTRACT

Members of subdivision 1 of the phylum *Acidobacteria* were grown at different pH values in a new medium formulation named PSYL 5, which includes sucrose as a carbon source and other compounds (such as  $\text{KH}_2\text{PO}_4$  and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ). Growth rate was nearly constant at pH 5.0 and declined at pH 3–4 and 6–7. However, it was found that effects involving good carbon/nitrogen ratios and pH on the growth of the members of *Acidobacteria* subdivision 1 were significant, and the strongest effect of these conditions was at pH 5.0. In addition, incubation time of 48, 72, 96 and 120 h was shorter than that described previously for members of *Acidobacteria* subdivision 1 on solid laboratory media.

**Keywords:** *Acidobacteria*; *Acidobacteria* subdivision 1; colony growth; growth in sucrose

## INTRODUCTION

*Acidobacteria* constitute one of the most dominant bacterial phyla in the soil ecosystem (Jones et al. 2009; Navarrete et al. 2013; Kielak et al. 2016). *Acidobacteria* is divided into 26 different subdivisions (Hugenholtz, Goebel and Pace 1998; Zimmermann et al. 2005; Barns et al. 2007). Recent ecological and physiological studies have reported evidence of *Acidobacteria* scavenging molecular hydrogen ( $\text{H}_2$ ) in the soil (Greening et al. 2015). However, some reports have described that until recently, no representatives of subdivision 1 were available for detailed study due to their apparent inability to grow in or on standard microbiological media (Zimmermann et al. 2005; Eichorst,

Breznak and Schmidt 2007; Ward et al. 2009). Culture-independent studies indicated that the phylogenetic breadth of *Acidobacteria* is as great as that of the phylum *Proteobacteria* (Eichorst, Breznak and Schmidt 2007). Most of the available data are mainly from studies based on cultivation-independent approaches such as next-generation sequencing of the 16S rRNA gene molecular marker. The composition of acidobacterial communities in soils has also been shown to vary with soil pH, organic carbon, soil nitrogen, C:N ratio (Foesel et al. 2014), factors linked to soil acidity (Navarrete et al. 2013) and nutrient availability (Navarrete et al. 2015). In general, the available data suggest that pH is one of the environmental variables that affect the abundance of different *Acidobacteria* subdivisions.

**Table 1.** Medium composition.

Ingredients	Medium composition (g l <sup>-1</sup> ) <sup>a</sup>	
	PSYA 5	PSYL 5*
KH <sub>2</sub> PO <sub>4</sub>	1.8	1.8
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2	0.2
Sucrose	30	30
Yeast extract	1	1
Agar	20	0

<sup>a</sup>Each medium was adjusted to a final pH of 5.0 with 1 M HCl.

\*Liquid medium.

Up to date, no much progress on the ecology and physiology of *Acidobacteria* subdivisions has been achieved. Studies based on *in vitro* observations are very scarce because *Acidobacteria* are very difficult to isolate and very slow growing bacteria in currently available culture media, e.g. low-nutrient solid media (Stott et al. 2008). Davis et al. (2005) have succeeded in detecting *Acidobacteria* on plates by a PCR-based surveillance method, using primers Acid31F (Barns, Takala and Kuske 1999) and 518R-GC (Muyzer, de Waal and Uitterlinden 1993) after 1 month of cultivation and George et al. (2011) reported that colony size kept increasing slowly with increasing incubation times (up to 1.5 years), except for subdivision 1 isolates. Nevertheless, novel strategies have been developed to improve the cultivation of *Acidobacteria* and other soil bacteria with low number of cultured representatives (Alain and Querellou 2009). Some types of modified medium formulations were reported in the literature to improve *Acidobacteria* recovery. For example the use of solidifier gelatin gum instead of agar (Janssen et al. 2002; Davis, Joseph and Janssen 2005), addition of polymeric growth substrate xylan instead of easily degradable carbon sources (Sait, Hugenholtz and Janssen 2002; Joseph et al. 2003), culturing with combinations of concentrations of aluminium potassium sulphate (Navarrete et al. 2013) or culturing with air enriched in CO<sub>2</sub> (Stevenson et al. 2004; Eichorst, Breznak and Schmidt 2007; Navarrete et al. 2013). Here for the first time we report the results of innovations required for the growth of *Acidobacteria* subdivision 1 strains in a short time. Furthermore, the aim was always to produce an abundant amount of cells, especially when the production of polymers such as exopolysaccharide and polyhydroxybutyrate is required.

## MATERIAL AND METHODS

### Bacterial culture conditions

Three *Acidobacteria* strains, WH120, WH15 (Valášková et al. 2009), 5B5 (KM979383), were used in this study. The strains used were isolated from wood in a stage of advanced decay, obtained from the collection of the Netherlands Institute of Ecology (NIOO-KNAW). The cultures are stored in VL-55 solid medium (Joseph et al. 2003) at -80 °C.

The modified solid media PSYA 5 (1.8 g KH<sub>2</sub>PO<sub>4</sub>, 0.2 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 30.0 g sucrose, 1.0 g yeast extract, 20 g agar) was adjusted to a final pH of 5.0 with 1 M HCl (Table 1). It is important that the following growth conditions should be pursued: (i) autoclave the medium for exactly 20 min at 121 °C and 1 kgf cm<sup>-2</sup> ≈ 0.968 atm; (ii) add 20 g of agar (Oxoid, Milan, Italy) per liter of medium in order to obtain a soft agar and therefore avoiding the wetting of the plates; (iii) adjust the final pH of the medium to 5.0; (iv) add cycloheximide (100 µg ml<sup>-1</sup>) to avoid the growth of

opportunistic fungi; (v) add the bacterial inoculum on recently prepared plates less than 48 h after solidification.

The inoculum of *Acidobacteria* was added on this modified media agar PSYA 5 by transferring a loopful from the stock culture on VL-55 media and the plates were incubated at 30 °C for 72 and 144 h.

### Inoculum preparation

A single colony of freshly prepared *Acidobacteria* culture grown in solid medium PSYA 5 was suspended in 250 µl of PSYL 5 liquid medium for 15 min, and then transferred for growth for 72 h at 30 °C on PSYA 5 agar plates. These cultures were then used for cell suspension (2 ml) onto the plate's surface from a 3-day-old culture.

### Colony morphology

Bacterial colonies were examined by photography using a Leica MZ75 stereomicroscope (Leica, Heerbrugg, Switzerland) equipped with a Leica DPD 250 camera (Leica, Heerbrugg, Switzerland) after 48, 72 and 96 h of growth in the dark, at 30 °C, on solid agar medium PSYA 5. The quality of the images allowed for determining the cell size, using the ImageProPlus image analysis software (Media Cybernetics, Inc., Rockville, MD, USA).

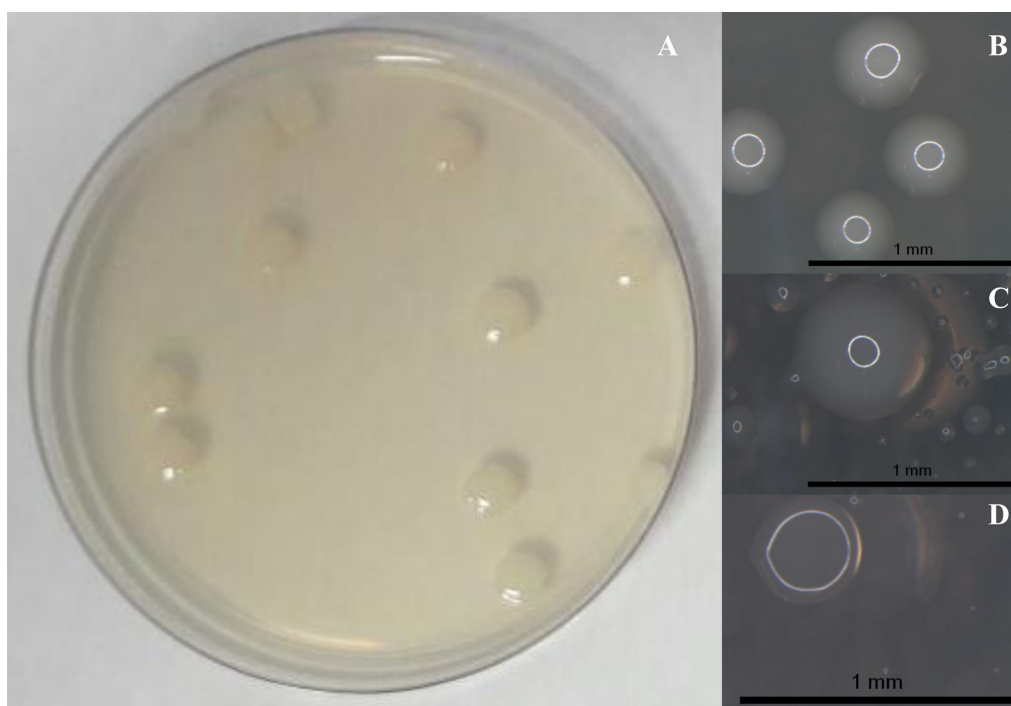
### Growth curves

Growth was monitored by measurements of optical density at 600 nm (OD<sub>600</sub>) with an Eppendorf photometer (Eppendorf, Hamburg, Germany). For the growth curves, 50 ml of PSYL 5 medium was used. Cell suspensions were transferred in the same medium (PSYL 5) until the culture reached an OD<sub>600</sub> of 0.01 (inoculum cells). The number of bacteria (colony-forming units (CFU) per milliliter) on the inoculum at this time was estimated by plating the diluted samples on solid agar medium PSYA 5. The colonies of WH120 and WH15 strains were counted after 72 h of incubation at 30 °C while the 5B5 strain was counted after 120 h, and adjusted according to the dilution factor.

Cultures were grown under aeration at a constant rotation rate of 150 rpm. Every 24 h, samples were measured in triplicate at a wavelength of 600 nm. After the measured turbidity, the culture was subjected to centrifugation (12 000 × *g* for 40 min at 4 °C) to separate the cells from the supernatant. The pH of the cell-free medium supernatant was measured by Radiometer, pHmeter 28 (Radiometer Analytical SAS, Villeurbanne cedex, France). The recovered cells (cell biomass) were suspended in saline buffer (0.85% NaCl), centrifuged (12 000 × *g* for 15 min at 4 °C), and oven dried at 50 °C until constant weight. The amount of dry biomass was evaluated by gravimetric measures. This procedure was developed in triplicates for 6 days of growth.

### Effect of pH on growth

To determine the pH effect on the growth rate, 5B5, WH15 and WH120 strains were grown at 30 °C using pH values from 3.0 to 7.0. The PSYL 5 liquid medium was used to test the pH effect on growth with 3% sucrose as a carbon source. Culture tubes (Sigma-Aldrich Corporation, St. Louis, MO, USA) containing 5 ml of medium were incubated in an orbital shaker at 150 rpm at 30 °C for 144 h. The optical density at 600 nm was monitored at 72 h and 144 h of incubation with an Eppendorf photometer.



**Figure 1.** Colonies of WH120 (A and B), WH15 (C) and 5B5 (D) strains on PSYA 5 medium after 72 h of cultivation at 30 °C for WH120 and WH15, and after 96 h of incubation for the 5B5 strain. The scale bar in the photomicrographs represents 1 mm for all panels.

In all experiments, the DNA of the bacterial cells was PCR amplified using acidobacterial primers Acid31F (Barns, Takala and Kuske 1999) and the universal bacterial primer RD1 (Weisburg et al. 1991), and the PCR products were sequenced.

### Statistical analysis

The effect of pH on growth were performed three times in triplicate. One-way analysis of variance was used to determine the differences and Tukey's test was used to calculate statistical significance, using the SAS package, Version 8.2 [Raleigh, NC, USA (SAS Institute Inc. 2001)]. A probability value  $P < 0.05$  was considered to be statistically significant.

## RESULTS AND DISCUSSION

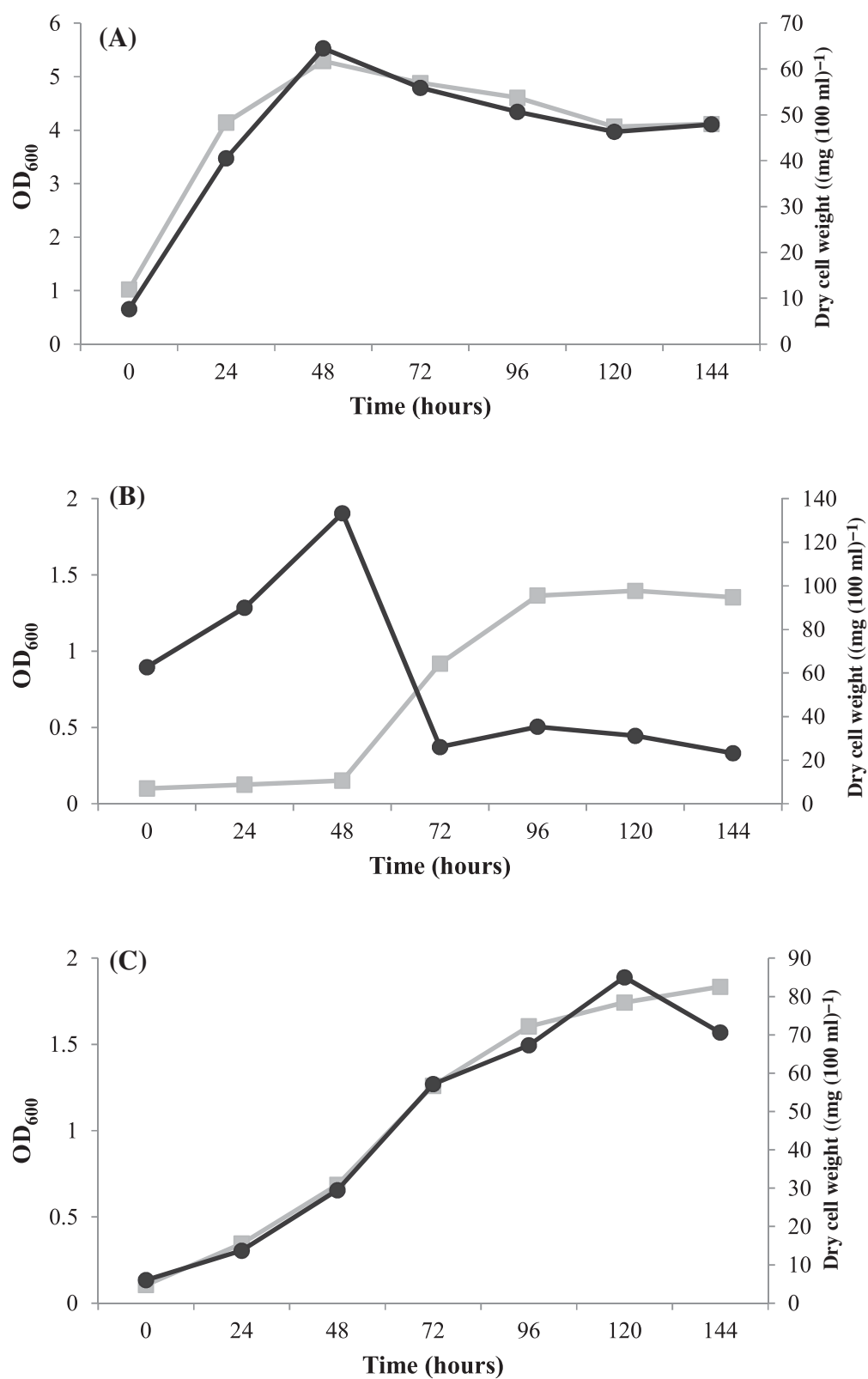
### Cultivation and characterization of *Acidobacteria* on plates

In order to improve the growth of *Acidobacteria* in laboratory conditions, a different growth medium supplemented with sucrose (10–30 g l<sup>-1</sup>, pH 5) as a carbon source, was tested. The growth of three *Acidobacteria* subdivision 1 (WH120, WH15 and 5B5) strains on solid culture medium PSYA 5 is shown in Fig. 1. Under aerobic conditions and on a solid medium, the WH120 strain produced several bacterial colonies less than 0.3 mm in diameter, whereas WH15 strain had convex and very smooth colonies of 0.5 mm in diameter after 72 h of incubation. However, both WH120 and WH15 strains showed visible colonies on the surface of the agar plates only after approximately 2–3 days. The colonies' colors were milk-white, smooth and showed a homogeneous consistency. Strain 5B5 formed very small colonies, with diameters ranging between 0.1 and 0.4 mm, after 72 and 96 h, respectively, with dried and pale-cream colonies and a central depression (Fig. 1C). The pale cream color of the 5B5 strain can be justified

by the dryness of the colonies and also due to the yeast extract culture medium high content. All colonies of WH120, WH15 and 5B5 strains presented cell morphology typical of Gram-negative bacteria (Fig. 1).

It was found that *Acidobacteria* grow very slowly, requiring several days to weeks to form visible colonies on Petri dishes. However, our incubation times of 48, 72, 96 and 120 h were relatively very short compared to 21–36 days (Watve et al. 2000), 14–21 days (Eichorst, Kuske and Schmidt 2011) and 168 days (Davis, Sangwan and Janssen 2011) to obtain visible colonies on solid medium.

Several strains of *Acidobacteria* were successfully recovered using a simple strategy based on the following: the dilution of nutrients in a culture medium to at least 10 times less than that found in the original concentration, dilutions of the inoculum to avoid high cell concentration, use of complex polysaccharides as carbon sources, longer incubation times, and high-throughput methods such as the 'plate-wash' PCR to detect targeted groups (Janssen et al. 2002; Sait, Hugenholtz and Janssen 2002; Stevenson et al. 2004; Davis, Joseph and Janssen 2005). The most used medium for *Acidobacteria* isolation, growth and maintenance is VL-55 medium solidified with gellan gum, pH 5.5 (Sait, Hugenholtz and Janssen 2002; Joseph et al. 2003), that is a chemically defined culture medium that contains xylan as the sole carbon source, and amended with a vitamin solution (Janssen et al. 1997), as well as trace element solution SL-10 (Widdel, Kohring and Mayer 1983). de Castro et al. (2013) evaluated *Acidobacteria* growth ability by using polysaccharides as carbon sources and several isolates grew in the pentoses (i.e. arabinose and xylose) and hexoses (i.e. dextrose and fructose), except AB20 and AB39 isolates, what were not able to grow well on fructose. However, the authors observed colony-forming units after 2 weeks at 25 °C, and the fully developed colonies was observed only after 4 weeks. Both these bacteria were very slow growing and formed visible colonies only after long time of incubation or had



**Figure 2.** Growth curves of *Acidobacteria* WH120 (A), WH15 (B) and 5B5 (C) strains on liquid media PSYL 5 with sucrose as carbon source. Black circles, dry biomass; grey squares, optical densities measured at 600 nm.

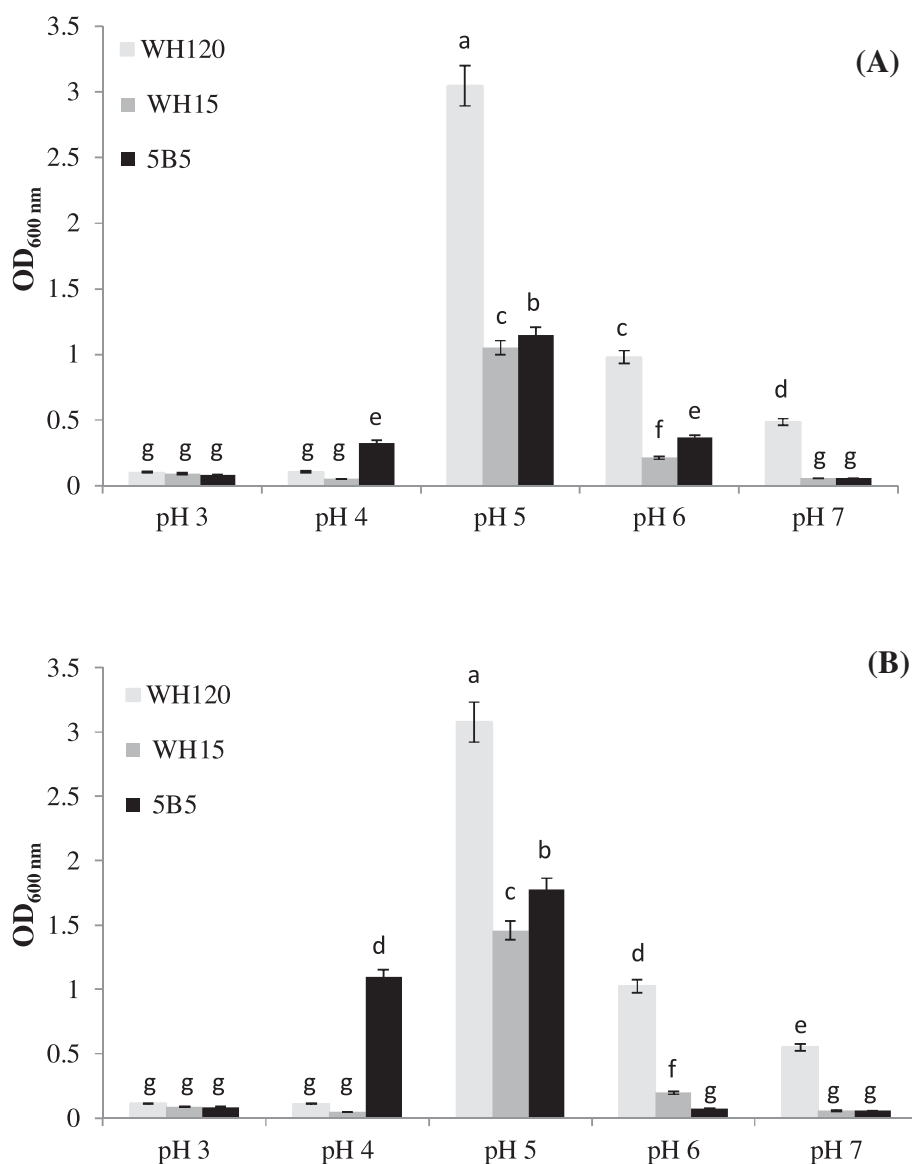
a very long lag period prior to initiation of growth. Generally, members of *Acidobacteria* have been difficult to grow and maintain in the laboratory. Data from the initial growth analysis of three strains suggest that at least these *Acidobacteria* (WH120, WH15 and 5B5) strains were capable of using sucrose as carbon source and other compounds (such as  $\text{KH}_2\text{PO}_4$  and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) of PSYL 5 medium. This medium used in our study might have beneficial effects on the initial growth of fastidious *Acidobacteria* strains of subdivision 1. As observed for the solid media experiments, the three *Acidobacteria* (WH120, WH15 and 5B5) had better growth on modified media agar PSYA 5 than VL-55 medium (data not shown), and the colony-forming had been observed after only 3 days of incubation. This result confirms that *Acidobacteria* would exhibit growth stimulation by the PSYL 5 liquid medium described here. Generally there are the influences of environmental factors such as carbon source and its concentration, and carbon to nitrogen ratio, as well as temperature and pH on the growth rate. Under the conditions used in these experiments, the highest initial C content and the lowest initial N

content in the culture media resulted in high specific growth rates that allowed the fastest specific growth rate after incubation at 30 °C and pH 5.5.

### Growth curve of the acidobacteria strains

The growth of three bacterial strains, members of the phylum *Acidobacteria* (affiliated with subdivision 1) named WH120, WH15 and 5B5, on PSYL 5 liquid medium is shown in Fig. 2. As can be seen, optical density measurements yielded similar growth curves, with lag, log and stationary phases clearly defined for all strains. However, the strain 5B5 and WH120 grew better on PSYL 5 medium than WH15, whereas the 5B5 strain grew significantly slower than WH120 and WH15 strains in PSYL 5 liquid medium with sucrose as carbon source.

The number of bacteria ( $\text{CFU ml}^{-1}$ ) in the inoculum at the inoculation time were estimated by plating the diluted samples on modified media agar PSYA 5. The results show that WH120



**Figure 3.** Effects of initial pH on the inhibition of *Acidobacteria* growth after 72 h (A) and 144 h (B) of incubation on liquid medium at 30 °C. Each value represents the mean of three replicates. Different letters above the bars indicate a significant difference ( $P < 0.05$ ).



had  $1.65 \times 10^8$  cel ml<sup>-1</sup>; WH15 had  $1.5 \times 10^8$  cel ml<sup>-1</sup>; and 5B5 had  $2.3 \times 10^8$  cel ml<sup>-1</sup>, with the dilution factor times a 0.1 ml inoculum of the  $10^{-5}$  dilution. WH120 samples with a high OD needed to be diluted prior to starting at time 0 of the bacterial growth curve experiment. However, for the final OD values it was necessary to multiply the diluted OD by the dilution factor. The lag time was not influenced by the initial cell concentration on the cell suspension. This phase is when the cells are adapting the metabolism to the new culture environment and prepare for cell proliferation (Tortora, Funke and Case 1986). After 120 h of incubation, cells enter the stationary phase. Our results demonstrate that yeast extract plus sucrose, as carbon source, provide nutrition that is sufficient for *Acidobacteria* cells to grow. Liquid acidic conditions (pH 5.0) and temperatures kept at 30 °C are also optimum. From Fig. 2B, it can be seen that there was a difference in biomass after 48 h of incubation at 30 °C, during the growth of the WH15 strain.

### Effect of pH

One additional experiment has been conducted to determine the effect of pH on the growth rate. This was done to confirm whether the pH would play an important role in *Acidobacteria* assembly, especially members of subdivision 1, since the pH apparently has a strong influence on the development of their colonies (Sait, Davis and Janssen 2006). Cells were inoculated into the PSYL 5 liquid medium made up with several pH values (3, 4, 5, 6 and 7) and growth curves were measured. In this experiment, PSYL 5 medium contained sucrose with pH values ranging from 5 to 10; the cultures were incubated under aerobic conditions at a temperature of 30 °C with shaking at 150 rpm.

The measured pH values of the cultures were constant and very stable during the whole experiment. The pH influenced the development of bacteria growth for 5B5, WH15 and WH120 strains in liquid medium (PSYL 5). Growth occurred only when sucrose was added and pH adjusted to 5.0. Interestingly, from these experiments it was possible to observe the preference of the strains for acidic media. In this study, the optimum pH value for the best growth of three *Acidobacteria* strains was found to be 5.0, since maximum values for growth rate were obtained at this pH. It was possible to see from the results that a better growth was observed for WH120 strain over a pH range varying from 5.0 to 7.0 with highest growth rate at pH 5.0, and for WH15 and 5B5 strains a good growth was observed over a pH range from 5.0 to 6.0 (Fig. 3). The highest limiting conditions were recorded at pH values lower than 4 and above 7. A predominance of *Acidobacteria*, especially from subdivision 1, has been detected in low pH conditions, e.g. a medium with pH 5.5 yielded significantly more colonies than a medium with pH 7.0 (Sait, Davis and Janssen 2006).

Pankratov et al. (2008) showed that peat-inhabiting *Acidobacteria* were adapted to more acidic conditions and low temperatures (between 2 and 32 °C, with an optimum at 15–22 °C), grew between pH 3.1 and 7.8, with an optimum at pH 3.5–4.5. Several reports suggested that low pH may allow more successful isolation of at least members of subdivision 1 from soil and other habitats (Stevenson et al. 2004).

Petrackova et al. (2010) demonstrated previously that the pH adaptation resulted in a modification of the fatty acid content of cellular membranes that significantly influences both the lipid-chain order and its dynamics. The pH of the medium can exert a selective pressure, and only those microorganisms with adaptive mechanisms are able to grow. Most proteins and other biologically important factors would assist bacteria to adapt to

environmental acidic conditions. This conclusion is consistent with the best growth of bacteria adapted to pH 5.0. One might say that the possible reason for this is related to cell specialization for cultivation in acidic conditions: the lipid order increases and membrane dynamics decreases as compared to pH 7.0 conditions.

The purpose of this study was to optimize medium culture for *Acidobacteria* subdivision 1 strains. Therefore, this semi-defined medium was achieved with sucrose and yeast extract as C and N sources, respectively, and a pH of 5. This medium will be used for cultivation of the acidobacterial communities to supply them for the initial growth and adaptation in the laboratory. In addition, these optimized cultivation conditions can be tested for the fast growth of members of subdivision 1 in diverse habitats isolated from.

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**Conflict of interest.** None declared.

### REFERENCES

- Alain K, Querellou J. Cultivating the uncultured: limits, advances and future challenges. *Extremophiles* 2009;13:583–94.
- Barns SM, Cain EC, Somerville L et al. *Acidobacteria* phylum sequences in uranium-contaminated subsurface sediments greatly expand the known diversity within the phylum. *Appl Environ Microbiol* 2007;73:3113–6.
- Barns SM, Takala SL, Kuske CR. Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. *Appl Environ Microbiol* 1999; 65:1731–7.
- Davis KER, Joseph SJ, Janssen PH. Effects of growth medium, inoculum size, and incubation time on the culturability and isolation of soil bacteria. *Appl Environ Microbiol* 2005;71: 826–34.
- Davis KER, Sangwan P, Janssen PH. *Acidobacteria*, *Rubrobacteridae* and *Chloroflexi* are abundant among very slow-growing and mini-colony-forming soil bacteria. *Environ Microbiol* 2011;13: 798–805.
- de Castro VH, Schroeder LF, Quirino BF et al. *Acidobacteria* from oligotrophic soil from the Cerrado can grow in a wide range of carbon source concentrations. *Can J Microbiol* 2013;59: 746–53.
- Eichorst SA, Breznak JA, Schmidt TM. Isolation and characterization of soil bacteria that define *Terriglobus* gen. nov., in the phylum *Acidobacteria*. *Appl Environ Microbiol* 2007;73:2708–17.
- Eichorst SA, Kuske CR, Schmidt TM. Influence of plant polymers on the distribution and cultivation of bacteria in the phylum *Acidobacteria*. *Appl Environ Microbiol* 2011; 77:586–96.
- Foesel BU, Nägele V, Naether A et al. Determinants of *Acidobacteria* activity inferred from the relative abundances of 16S rRNA

- transcripts in German grassland and forest soils. *Environ Microbiol* 2014;**16**:658–75.
- George IF, Hartmann M, Liles MR et al. Recovery of as-yet-uncultured *Acidobacteria* on dilute solid media. *Appl Environ Microbiol* 2011;**77**:8184–8.
- Greening C, Constant P, Hards K et al. Atmospheric hydrogen scavenging: From enzymes to ecosystems. *Appl Environ Microbiol* 2015;**81**:1190–9.
- Hugenholtz P, Goebel BM, Pace NR. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol* 1998;**180**:4765–74.
- Janssen PH, Schuhmann A, Mörschel E et al. Novel anaerobic ultramicrobacteria belonging to the *Verrucomicrobiales* lineage of bacterial descent isolated by dilution culture from anoxic rice paddy soil. *Appl Environ Microbiol* 1997;**63**:1382–8.
- Janssen PH, Yates PS, Grinton BE et al. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. *Appl Environ Microbiol* 2002;**68**:2391–6.
- Jones RT, Robeson MS, Lauber CL et al. A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. *ISME J* 2009;**3**:442–53.
- Joseph SJ, Hugenholtz P, Sangwan P et al. Laboratory cultivation of widespread and previously uncultured soil bacteria. *Appl Environ Microbiol* 2003;**69**:7210–5.
- Kielak AM, Barreto CC, Kowalchuk GA et al. The ecology of *Acidobacteria*: moving beyond genes and genomes. *Front Microbiol* 2016;**7**:744.
- Muyzer G, de Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 1993;**59**:695–700.
- Navarrete AA, Diniz TR, Braga LPP et al. Multi-analytical approach reveals potential microbial indicators in soil for sugarcane model systems. *PLoS One* 2015;**10**:e0129765.
- Navarrete AA, Kuramae EE, de Hollander M et al. *Acidobacterial* community responses to agricultural management of soybean in Amazon forest soils. *FEMS Microbiol Ecol* 2013;**83**: 607–21.
- Pankratov TA, Serkebaeva YM, Kulichevskaya IS et al. Substrate-induced growth and isolation of *Acidobacteria* from acidic *Sphagnum* peat. *ISME J* 2008;**2**:551–60.
- Petrackova D, Vecer J, Svobodova J et al. Long-term adaptation of *Bacillus subtilis* 168 to extreme pH affects chemical and physical properties of the cellular membrane. *J Membr Biol* 2010;**233**:73–83.
- Sait M, Davis KER, Janssen PH. Effect of pH on isolation and distribution of members of subdivision 1 of the phylum *Acidobacteria* occurring in soil. *Appl Environ Microbiol* 2006;**72**:1852–7.
- Sait M, Hugenholtz P, Janssen PH. Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. *Environ Microbiol* 2002;**4**:654–66.
- SAS User's Guide, Version 8.2. Cary, NC, USA: SAS Institute Publications, 2001.
- Stevenson BS, Eichorst SA, Wertz JT et al. New strategies for cultivation and detection of previously uncultured microbes. *Appl Environ Microbiol* 2004;**70**:4748–55.
- Stott MB, Crowe MA, Mountain BW et al. Isolation of novel bacteria, including a candidate division, from geothermal soils in New Zealand. *Environ Microbiol* 2008;**10**:2030–41.
- Tortora GJ, Funke BR, Case CL. Microbiology. Menlo Park, CA, USA: Benjamin/Cummings, 1986.
- Valášková V, de Boer W, Gunnewiek PJK et al. Phylogenetic composition and properties of bacteria coexisting with the fungus *Hypholoma fasciculare* in decaying wood. *ISME J* 2009;**3**:1218–21.
- Ward NL, Challacombe JF, Janssen PH et al. Three genomes from the phylum *Acidobacteria* provide insight into the lifestyles of these microorganisms in soils. *Appl Environ Microbiol* 2009;**75**:2046–56.
- Watve M, Shejval V, Sonawane C et al. The 'K' selected oligotrophic bacteria: a key to uncultured diversity? *Curr Sci* 2000;**78**:1535–42.
- Weisburg WG, Barns SM, Pelletier DA et al. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 1991;**173**: 697–703.
- Widdel F, Kohring G, Mayer F. Studies in dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterisation of the filamentous gliding *Desulfonema limicola* gen. nov., sp. nov. and *Desulfonema magnum* sp. nov. *Arch Microbiol* 1983;**134**:286–94.
- Zimmermann J, Gonzalez JM, Saiz-Jimenez C et al. Detection and phylogenetic relationships of highly diverse uncultured acidobacterial communities in Altamira Cave using 23S rRNA sequence analyses. *Geomicrobiol J* 2005;**22**:379–88.