

Carbohydrate degrading bacteria closely associated with *Tetraselmis indica*: influence on algal growth

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Abstract

This investigation was conducted in order to examine the interactions between an algal species, *Tetraselmis indica* (a new species described, manuscript under consideration elsewhere), collected in the wild and strains of bacteria with which it is closely associated. Three bacterial strains were isolated and sequence analysis of the 16S rDNA indicated that the organisms belonged to the genera *Pseudomonas*, *Acinetobacter* and *Ruegeria*. Morphologies of the bacterial strains were studied using epifluorescence microscopy and scanning electron microscopy. Re-association experiments were conducted with axenic cultures inoculated with the three bacterial strains in concentrations comparable to natural conditions, and the effect of each bacterial population on the growth of *T. indica* was determined. *Tetraselmis indica* exhibited differential growth success with the various bacterial cultures, and in particular *Acinetobacter* sp. was observed to promote growth of the algae. These experiments revealed that microbes associated with the alga differentially influence algal growth dynamics. Bacterial presence on the cast off cell wall products of alga suggested the likely utilisation of algal cell wall by bacteria. The bacterial strains were tested for carbohydrate metabolism using various sugars and screened for carbohydrase activity. Bacterial strains were found to produce carbohydrases for degradation of polysaccharides generally present in the cell wall of *T. indica* (glucans, galactans, galactomannans and pectins), whereas no such utilisation was observed for other wall substrates such as cellulose, arabinoxylan, rhamnogalacturonan). The *Pseudomonas* sp. and the *Acinetobacter* sp. showed carbohydrase activity with Glucans, galactans, galactomannans and pectin, whereas the *Ruegeria* sp. showed much less carbohydrase activity and only with pectin. The carbohydrate utilisation studies using artificial substrates suggested the potential utilisation of cast-off algal cell wall products.

INTRODUCTION

Phytoplankton release considerable amounts of organic components into the extracellular medium, most of which are utilised by bacteria (Cole et al. 1988, Williams 1981, Coveny 1982, Coveny & Wetzel 1989, Ducklow & Carlson 1992, Kormas 2005). Consequently, heterotrophic bacteria are often found in close associations with algae, which are able to provide a favourable habitat for these bacteria within aquatic ecosystems (Paerl & Pinckney 1996, Caldwell 1977). The possible role of bacteria in enhancing the growth of algae has been documented by several investigators (Abalde & Herrero 1992, Giorgio et al. 1997, Moreno & Laine, 2004, Kluijver et al. 2010). Lange (1967, 1970, & 1971) has proposed that bacteria may be a significant source of CO₂ for algal growth during periods of carbon limitation.

The taxa of bacteria that can be cultured from samples of phytoplankton are often different from those that can be cultured from water samples (Simidu et al. 1971, Kogure et al. 1981, Cole 1982). Specific bacteria are habitually associated with phytoplankton and are highly adapted for extracellular algal products utilisation (Bershova et al. 1968, Kuentzel 1969, Bell & Mitchell 1972, Bell & Sakshaug 1980, Meffert & Overbeck 1985, Maurin et al. 1997, Aota & Nakajima 2001, Huang et al. 2004, Zifeng et al. 2009). Heterotrophic utilisation of algal products can be expected to be a function of both the activity of the heterotrophs and of the suitability of the excreted material (Coveny 1982). In addition to the exudation of organic components (Mague et al. 1980, Bjornsen 1988, Mykkestad et al. 1989, Obernosterer & Herndl 1995) algal components released through cell lysis (Brussard et al. 1988) and the cast off theca (Lewin 1958, Gooday 1971, Becker 1991) may be an important carbon source for bacteria.

This current investigation was conducted in order to examine the type of interactions under controlled conditions between an algal species collected from the wild and strains of bacteria with which it is closely associated. The alga used for this purpose was *T. indica* (Arora et al., manuscript in submission) collected from a salt pan in Goa, India. During the non-motile phase of this alga, new walls develop and old walls are cast off and accumulate as concentric rings surrounding the cell.

The bacterial strains isolated from *T. indica* were grown in combination with axenic cultures of *T. indica*. An examination of the interactions between the bacteria and the algal species was conducted. In order to confirm the degradation and utilisation of cast off algal cell wall products by the bacteria, bacterial utilisation of carbohydrates was tested.

MATERIALS AND METHODS

Isolation of culturable planktonic and epiphytic bacteria by growth on selective media

Tetraselmis indica cultures were maintained in F/2 media (Guillard & Ryther 1962) without silicates, at 25°C, photon flux density 80 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and a 16:8 h L: D cycle. 500 ml of *T. indica* culture (in sterile F/2 algal culture media) was sonicated using a sonicator (Ultrawave U300H with ultrasonic power 35 W, and operating frequency 44 KHz) for 5 minutes and filtered through a sterile GF/C (1.2 μm), Whatman glass fibre filter. The filtrate was again filtered through a 0.2 μm Whatman membrane filters whilst under vacuum. The 0.2 μm filters with the bacteria present were then washed using 2 ml of sterile sea water and mildly sonicated (for 3 minutes). Serial dilutions (10^{-1} - 10^{-6}) were prepared using sterile sea water. A 0.1 ml aliquot of each dilution was then plated onto marine agar (Difco) and on various selective media in triplicate under aseptic conditions. 0.1 ml of 0.2 μm filtrate and F/2 media were also plated as controls on marine agar and selective media. Marine agar was chosen as a generalised medium to grow all the culturable bacteria from algal culture. Depending on the source of isolation any micro or macro algae from sea water could contain culturable coliforms, vibrios, as well as other heterotrophic bacteria such as *Shewanella*, *Alteromonas*, *Planococcus* etc. therefore we chose to use Difco A-1 medium, suitable to detect any coliforms, TCBS is highly selective for vibrios, Pseudomonas isolation agar for isolating pseudomonas and Seawater agar media for any other heterotrophic bacteria.

1. A-1 Medium (Difco) for growth of coliforms including *Escherichia coli*.
2. Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS, Difco) for growth of *Vibrio* spp.
3. Pseudomonas isolation agar (Difco) for growth of pseudomonads.
4. Sea-water agar medium (SWA, DSMZ, 246) (Difco) for growth of heterotrophic bacteria.

Marine agar, TCBS, A-1 and SWA plates were incubated at room temperature (25° C in a temperature controlled building) for 24 h and the plates for selective pseudomonads growth at 37° C for 48 h.

Identification and phylogenetic analysis of bacteria

Three characteristic bacterial morphologies were identified from all the replicates of marine agar plates, representing diversity of bacteria associated with *Tetraselmis*. Single colony morphology of similar type was observed in each plate of A-1 Medium, Pseudomonas isolation agar medium and Sea-water agar. Single colonies from each plate were streaked for pure strain isolation. Identification of bacterial strains was performed by 16S rDNA sequence analysis. Amplification of the 16S rRNA gene sequences was performed using genomic DNA as the template, extracted using the standard alkaline-lysis protocol from whole

bacteria (Birnboim and Doly 1979). The 16S rRNA gene was PCR-amplified for all strains using universal prokaryotic primers: 27F 5'AGAGTTTGATCCTGGCTCAG3' (*E.coli* 27-47) and 1492R 5'TACGGTTACCTTGTTACGACTT3' (*E.coli* 1492-1514) (Lane 1991). The amplification reactions were carried out using a QIAGEN Fast Cycling PCR Kit and an Eppendorf Mastercycler PCR machine. Dye terminator sequencing, using the same primers as in the amplification step, and an Applied Biosystems 3730xl DNA Analyzer at Newcastle University were used to obtain the nucleotide sequences. 16s rDNA sequences were determined and then compared with those available in the National Centre for Biotechnology Information (NCBI) database using the BLASTN algorithm (Altschul et al. 1990), and the percentage identity was calculated. Consensus sequences were aligned using CLUSTALW2 (Hall 1999; Larkin et al. 2007). The TrN+I+G (Tamura-Nei) Model was selected using Mega version 5 (Tamura et al. 2011) and used as a model of nucleotide substitution for the phylogenetic inference of each sequence by the maximum likelihood method. Neighbour joining and maximum parsimony trees were also inferred using Mega. The sequences obtained have been submitted to NCBI GenBank under the accession numbers JF828049, JF828050 and JF828051.

Morphological study of bacteria using epifluorescence microscopy and scanning electron microscope

Cultured cells were sampled during the late exponential growth phase. Cells were stained using SYBR Gold stain at a working dilution of 10 $\mu\text{l ml}^{-1}$ (Molecular Probes, Invitrogen, 1:100 dilution in distilled water) and observed under epifluorescence microscopy. For scanning electron microscope (SEM), cells were fixed in 2% glutaraldehyde (TAAB Laboratory Equipment), in cacodylate and sea water. Cells were allowed to settle on poly-l-lysine coated coverslips before placing the coverslips in a holder and dehydrating the cells in an ethanol series (10 min each in 25%, 50% and 75% followed by 15 min in 100% twice). The coverslips were dried in a critical point dryer (BalTec Ltd, Reading, UK) and subsequently mounted on stubs with a silver DAG and carbon disc (Agar Scientific, Stansted, Essex). Finally, the cells were sputtered with gold (using a Polaron SEM coating unit) and observed using a Stereoscan S40 Scanning Electron Microscope (Cambridge Instruments, UK) at the Electron Microscopy Unit of the School of Biomedical Sciences, Newcastle University.

Study of phytoplankton axenic culture growth with different types of bacteria

(1) Preparation of axenic and xenic culture of the phytoplankton: *T. indica* cells were washed (5 times) with sterile seawater before and after mild sonication (3 min) to remove closely adhered bacterial cells. Cultures were incubated in carbenicillin (final concentration 0.02 g L⁻¹) for 24 hrs and were given a sterile wash. Cultures were recovered for 6 days by incubating in sterile F/2 algal culturing medium (Guillard and Ryther 1962) at 20°C before being incubated in fresh sterile F/2 medium containing 0.1 g L⁻¹ cefotaxime

and kept for a further 24 hrs at 20°C. Cultures were given 5 repeated sterile washes and transferred to antibiotic-free F/2 medium. The purity of the algal cultures was tested by three methods: first by the spread plate method using Zobell marine agar (Himedia); secondly, microscopically by fluorochrome staining using 4-6 diamidinophenyl indole (DAPI) and acridine orange; and third by flow cytometry using SYBR Gold fluorescent stain (Molecular Probes, Invitrogen) and a BD FACS Aria II flow cytometer. For flow cytometry samples were run at a rate of 10 µl min⁻¹ on a FACS Aria II flow cytometer (Becton Dickinson) equipped with a 488 nm excitation laser and a standard filter setup. To create xenic cultures of alga (microalgal cultures containing bacteria), axenic cultures (75 ml each) were inoculated directly at the start of the experiments with a mixed community of bacteria (presumably culturable as well as non-culturable) present in the original isolate. The mixed community of marine bacteria was obtained from the sonicated culture at the end of the exponential phase by filtration through a sterile GF/C, Whatmann glass fibre filter (Brussard et al. 1998). The filtrate was then used as the bacterial inoculum and was added into axenic culture for preparation of xenic cultures of *T. indica*. The mixed bacterial community therefore consisted of a group of bacterial species originally present in the culture and adapted to the specific culture conditions. The concentration of bacteria at the start of the experiments was between 0.45 - 0.6 × 10⁶ cells ml⁻¹. A control was prepared using the axenic culture which received 5 ml of the 0.2 µm filtrate containing no bacteria.

(2) Estimation of bacterial and algal population by counting through light microscopy epifluorescence microscopy and flow cytometry: For the estimation of bacterial biomass, all cultures were subjected to mild sonication and filtered through GF/C, 1.2 µm filters before measuring the optical density at 590 nm. Bacterial cell counts were obtained using epifluorescence microscopy and the acridine orange direct count method (Hobbie et al. 1977). Cell counts were also calculated by staining the cells with SYBR Gold (Molecular Probes, Invitrogen) and using flow cytometry.

To study the effect of each bacterial type on the phytoplankton population in terms of cell numbers, growth dynamics of axenic culture and xenic culture were studied under standard culture conditions. A single colony from each bacterial plate was suspended in 1 ml of sterile sea water which was serially diluted to give a range of 10⁻¹ to 10⁻⁶ cells ml⁻¹. Each strain of bacteria was prepared at a density of 3.2 × 10⁶ cells ml⁻¹. 5 ml of each bacterial inoculum was added to 75 ml of axenic culture to give a final bacterial concentration of 0.4 × 10⁶ cells ml⁻¹, a comparable concentration to those of xenic cultures. Total cell counts were performed using microscopy and phytoplankton growth was considered as being in the stationary phase when the cell counts did not differ significantly on three successive days. Algal growth during exponential, stationary and senescence phases was studied using cell counts calculated with a haemocytometer. For both xenic and axenic cultures, specific growth rates of the 10th to 18th day (exponential phase) of the experiment were calculated according to Levasseur et al (1993) using the following formula and were averaged.

$$K' = \text{Ln} (N2 / N1) / (t2 - t1)$$

Where N1 and N2 = cell counts on days (t1) and (t2) respectively

A comparison of dead cell counts of axenic culture in combination with various bacteria was performed using SYTOX Green stain (Molecular Probes, Invitrogen) and a BD FACS Aria II flow cytometer. Samples were run at a rate of 10 $\mu\text{l min}^{-1}$ on a FACS Aria II flow cytometer (Becton Dickinson) equipped with a 488 nm excitation laser and a standard filter setup.

Carbohydrate utilisation (mono and di-saccharide)

Biochemical assays were performed on all three bacterial strains isolated to test their ability to metabolise various carbohydrates mentioned in Table 5.2, resulting in the formation of an acid and the production of carbon dioxide. The medium involved for these assays contained peptone (10 g), beef extract (1 g), NaCl (5 g), phenol red (0.018 g). The above mentioned components were dissolved in 500 ml distilled water and the volume made up to 1 litre. This broth was sterilized in autoclave at 120°C for 15 minutes. 10% of each sugar solution was prepared by adding 2 g in 20 ml distilled water and autoclaved below 100 °C. 4 ml of phenol red broth and 1 ml of each sugar solution was dispensed into tubes with Durham gas collecting tubes for gas detection. A sample of each of the assigned bacteria was aseptically inoculated into each of the phenol red broth tubes, using a loop.

Following incubation, carbohydrates that had been metabolised produced an acidic waste product causing the phenol red to turn yellow, indicating a positive reaction. In certain cases, acid production may be accompanied by the evolution of a gas (CO₂) which is visible as a bubble in an inverted tube.

Evaluation of the biodegradability of carbohydrate substrates (polysaccharides)

Eight types of biodegradable substrates Azo-Galactan, Azo-Carob Galactomannan, AZ-Rhamnogalacturonan, Azo-CM-Cellulose, Red Pullulan, Wheat Arabinoxylan, β -Glucan (Megazyme) and Pectin (Sigma) were used in this study (Table 2), which were added to the culturing media SWA at the concentration of 2.5 g L⁻¹. Pure colonies of each bacterium were streaked onto the formulated media in triplicate and incubated at 37°C for 48h. Cleavage of the chromogenic substrates by bacteria produced a halo-like precipitation zone around the bacteria indicating positive activity. Non-chromogenic substrates were stained using 1% congo red for 15 min and a similar halo-like precipitation was observed following destaining with 1 M NaCl in cases of positive activity.

RESULTS

Isolation, identification and phylogenetic analysis of bacteria

Three media, A-1, *Pseudomonas* isolation agar and SWA demonstrated three morphologically different bacterial colonies to be present. Similar colonies were observed on marine agar. TCBS agar did not yield any bacterial growth indicating the absence of vibrios in culture. Sequence analysis of 16S rDNA from duplicates indicated that the organisms isolated from the *T. indica* belonged to the *Acinetobacter*, *Ruegeria* and *Pseudomonas* genera. The *Pseudomonas* sp. showed maximum sequence similarity to *P. stutzerii* (98%), the *Acinetobacter* sp. to *A. haemolyticus* (98%) and the *Ruegeria* sp. to *R. atlantica* (97%) (Fig. 1 and supplementary information).

Morphologies with epifluorescence microscopy and SEM

The three isolates were observed by epifluorescence microscopy and scanning electron microscopy. Morphologies as observed under epifluorescence microscopy correlated well with those viewed by SEM. The *Acinetobacter* sp. was seen to be present in pairs (Figs. 2a and 2b) as expected for members of genus *Acinetobacter* (Carr et al. 2003), whilst the *Ruegeria* sp. was observed to be spherical in shape and readily formed chains (Figs. 2c and 2d). The *Pseudomonas* sp. demonstrated a rod-shaped morphology (Figs. 2e and 2f). *Pseudomonas* colonies were hard and dry with a characteristic wrinkled appearance as expected for *Pseudomonas stutzerii* (Lalucat et al. 2006). Occurrence of irregular polygon-like structures or concentric zones has also been noted as described earlier for *Pseudomonas stutzerii* (van Niel et al. 1952, Lalucat et al. 2006). All morphological features observed were typical of their genera.

Growth experiments of axenic algal and bacterial cultures

Total cell counts and dead cell counts of *Tetraselmis* in cultures of axenic, xenic and axenic alga in combination with each isolated bacterium, for a period of algal growth was studied (Figs. 3, 4 and 5). Bacterial cell concentrations in the xenic culture gradually increased with time, reaching a concentration of 32.8×10^6 on 28th day after inoculation. A rapid decline in total cell counts was recorded in the axenic compared to xenic cultures in the senescent phase of alga (Fig. 3). In the senescent phase, the total algal cell counts in the axenic culture with either the *Acinetobacter* sp. or the *Ruegeria* sp. were relatively high (Fig. 4) and the dead cell counts showed a significant reduction (Fig. 5). This was in contrast to axenic culture alone and axenic culture with *Pseudomonas* sp. which showed lower total cell counts (Fig. 4) and increased dead cell counts (Fig. 5). Significant cell lysis and hence a decrease in total cell counts was observed in axenic cultures during the senescent phase, whereas no such lysis was observed in case of xenic cultures. Presence of bacteria in xenic cultures led to increased specific growth rate of 0.20 compared to axenic cultures with a specific growth rate of 0.15 in the exponential phase. Presence of bacteria was observed on the discarded cell walls of alga in the xenic culture using SYBR Gold staining and epifluorescence microscopy (Fig. 6).

Carbohydrate utilisation (mono and disaccharide)

In the control tubes to which bacteria were not added, the carbohydrate substrate was not utilised and there was no subsequent change in the colour of the indicator or evolution of gas, indicating a negative reaction. Some bacterial strains possess enzymes that degrade peptones present in the media to amino acids. These in turn are enzymatically converted by oxidative deamination to ketoaminoacids before being metabolised through the Krebs cycle for energy production. These reactions release ammonia, which accumulates in the medium forming ammonium hydroxide and producing an alkaline environment, causing the colour of the media to alter from red to pink. Pink colour of the media was detected in the control tubes with no carbohydrates for *Pseudomonas* and *Reuveria*, indicating that these enzymes were present. The *Acinetobacter* sp. caused all the sugar solutions tested to turn yellow, indicating the formation of an acidic waste product following hydrolysis of the carbohydrate tested. The production of gas was observed only when sucrose was utilised as the carbohydrate source (Fig. 7). The *Pseudomonas* sp. and the *Reuveria* sp. were unable to utilise some of the tested carbohydrates (Table 1) and no gas production was observed by either of these two bacterial strains with any of the substrates.

Evaluation of the biodegradability of polysaccharides

The *Pseudomonas* sp. and the *Acinetobacter* sp. showed carbohydrase activity and formed a halo-like precipitation zone (Fig. 8) with four (Azo-Galactan, Azo-Carob Galactomannan, β -Glucan and Pectin) of the eight carbohydrate substrates tested (Table 2), whereas the *Ruegeria* sp. showed much less carbohydrase activity and only with pectin. Additionally, the *Pseudomonas* sp. also demonstrated a low level of activity of the enzyme dextrinase. The cell wall of *T. indica* has been shown to be made of a pectin-like material, with galactose and galacturonic acid as major components (Lewin 1958, Gooday 1971). Pectins can consist of either a polymer of unsubstituted polygalacturonic acid residues or a backbone of galacturonic acid and rhamnose residues that are attached to galactan and arabinan side chains. Production of carbohydrases such as pectinases, glucanases, galactanases and mannanases from various species of *Pseudomonas* and *Acinetobacter* has also been reported in earlier studies (Kathoda et al. 1979, Simonson et al. 1982, Braithwaite et al. 1995, Braithwaite et al. 1997, Aboaba 2009, Titapoka et al. 2007, Zheng et al. 2011).

DISCUSSION

This study has investigated the influence of bacteria on the cell growth and cell death kinetics of *Tetraselmis indica* under culture conditions. The study showed that in contrast to xenic culture, reduced cell growth and enhanced mortality were recorded for axenic culture. Bacterial inoculation into cultures of *T. indica* had a pronounced effect on algal growth, which either decreased (as observed with the *Pseudomonas* sp.) or increased (as observed with the *Acinetobacter* sp.). Following inoculation with the *Acinetobacter* sp. and *Ruegeria* sp., prolonged survival was observed. Bacterially regenerated carbon from the discarded cell

walls was very likely utilised by the cultures during the senescent phase, thereby prolonging the survival of the *T. indica* population. This reinforces the concept that bacteria can enhance the growth of algae.

Another interesting analysis was that the xenic culture which contained both culturable and non-culturable bacteria demonstrated less growth compared to the growth associated with the *Acinetobacter* sp. alone. Therefore, there could be internal competition within the bacterial community that results in this reduced growth condition of the algae. In the xenic cultures bacteria were found to be attached to the discarded cell walls (Fig. 6). The cell wall of *T. indica* has been shown to be made of a pectin-like material, with galactose and galacturonic acid as major components (Lewin 1958, Gooday 1971). Galacturonic acid and some other 2-keto sugar acids impart an acidic character to the cell walls of *T. indica* (Becker 1991, 1998). Experimental results of the current study on the bacterial utilisation of different carbohydrate substrates indicated which carbohydrate substrates these bacteria depend on for their survival. Bacterial strains were found to produce carbohydrases for degradation of polysaccharides generally present in the cell wall of *T. indica* (glucans, galactans, galactomannans and pectins), whereas no such utilisation was observed for other wall substrates such as cellulose, arabinoxyln, rhamnogalacturonan). Since the *Acinetobacter* sp. and the *Pseudomonas* sp. were able to utilise the same substrates at almost equal levels, as indicated by the carbohydrate utilisation results, this suggests that they may compete for the same substrates within the cell wall of *T. indica*. Importantly, when the alga was grown with only the *Pseudomonas* sp. present, growth of the alga was inhibited leading to a prolonged lag phase and resumed only when the algal culture was in its exponential phase, and again arrested when the alga reached the senescent phase. Therefore, the speculation is that the *Pseudomonas* sp. has a negative influence on algal growth, notably it could be toxic for cell culture, and this bacterium is able to survive only as a competitor with the other associated bacteria in the cell wall environment. Even though the *Ruegeria* sp. enhanced the survival of the alga, after the senescent phase there was a neutral effect on the culture growth during the lag phase. Hence, it can be concluded from this study that each bacterium associated with *T. indica* demonstrates a differential influence on algal growth.

As can be seen in this study, these algal associated bacteria are a potential source of useful carbohydrases and could be used to prevent the formation of biofilms and in applications involving biofilm dispersal due to their polysaccharide degrading capability (Kaplan et al. 2003, Ramasubbu et al. 2005). In addition, the *Acinetobacter* sp. could also be used in microalgal aquaculture to increase growth of algae. More widely, this study reinforces the ecological importance of bacteria in marine biogeochemical cycles, in hydrolysing organic matter and in influencing the dynamics of phytoplankton.

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FIG. AND TABLE LEGENDS

Fig. 1- Phylogeny of 3 bacterial strains based on 16s rDNA sequences showing the position of bacteria in relation to the closest species of their respective genera. The phylogenetic tree shown was inferred by the maximum likelihood method based on the Tamura and Nei (1993) model of DNA substitutions

Fig. 2 – Morphology of the isolated bacteria. Panels A and B - epifluorescence and SEM micrograph of the *Acinetobacter* sp. showing bacteria present in pairs; Panels C and D - epifluorescence and SEM micrograph of the *Ruegeria* sp. showing its spherical morphology; Panels E and F - epifluorescence and SEM micrograph of the *Pseudomonas* sp. showing its rod shaped morphology.

Fig. 3- Variations of total algal concentrations (primary Y axis), percentages of dead cells and bacterial cell concentrations (secondary Y axis).

Fig. 4 - Total algal cell concentrations for axenic, xenic and axenic cultures inoculated with each of the bacterial species isolated. Values are means of triplicates \pm SE.

Fig. 5 – Plot of Dead cell concentrations from the experiment described in Fig. 3 for axenic, xenic and axenic cultures inoculated with each of the bacterial species isolated. Values are means of triplicates \pm SE.

Fig. 6- A: Epifluorescent micrograph showing the attachment of bacteria to the discarded cell wall of *T. indica*, solid arrow marks are pointing towards the cell wall and dotted arrow marks showing bacterial cells. B: Phase contrast micrograph of the discarded and disintegrating cell wall showing the presence of bacteria, solid arrow marks pointing towards disintegrating cell wall and bacteria and the dotted arrow marks showing live algal cell.

Fig. 7 - Utilisation of sucrose by the isolated *Acinetobacter* sp. The tube on the left is the control and the tube of the right demonstrates the colour change in the media following utilisation of the sucrose and the production of gas in the inverted tube.

Fig. 8 - Degradation of carbohydrates by the three bacterial species isolated. Ps, *Pseudomonas* sp., Ah, *Acinetobacter* sp., Ra, *Ruegeria* sp. Panel A – activity of endo-1,4- β -galactanase when azo-galactan was the substrate, Panel B – activity of β -glucanase when β -glucan is the substrate.

Table 1 – Utilisation of different carbohydrates by the three bacterial species isolated. + indicates the sugar was utilised and - that it was unable to be utilised.

Table 2 – Carbohydrate substrate utilisation by the three bacterial species isolated. - indicates no degradation of the substrate whilst + indicates the degradation of the substrate.

Fig. 1

Phylogenies:

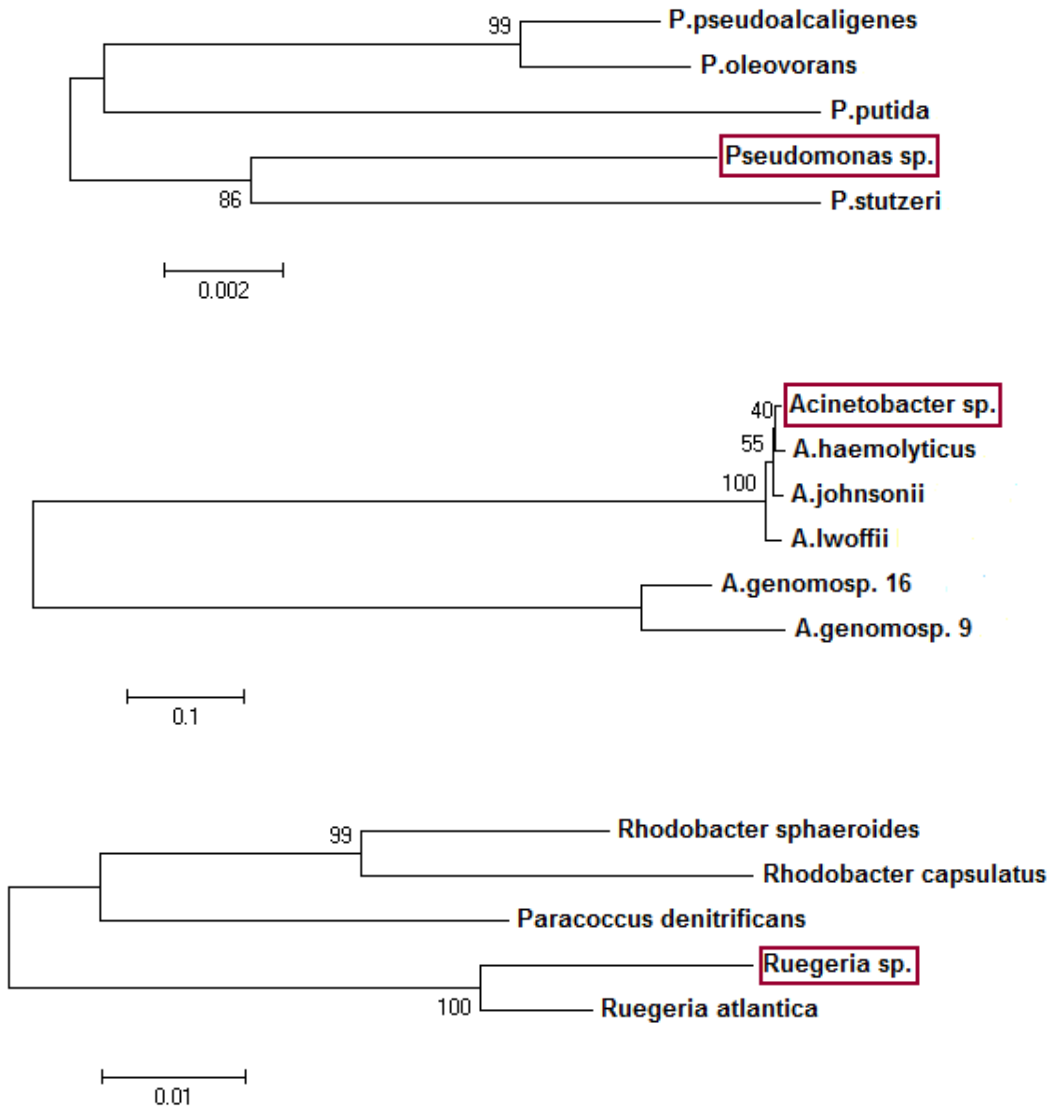


Fig. 2

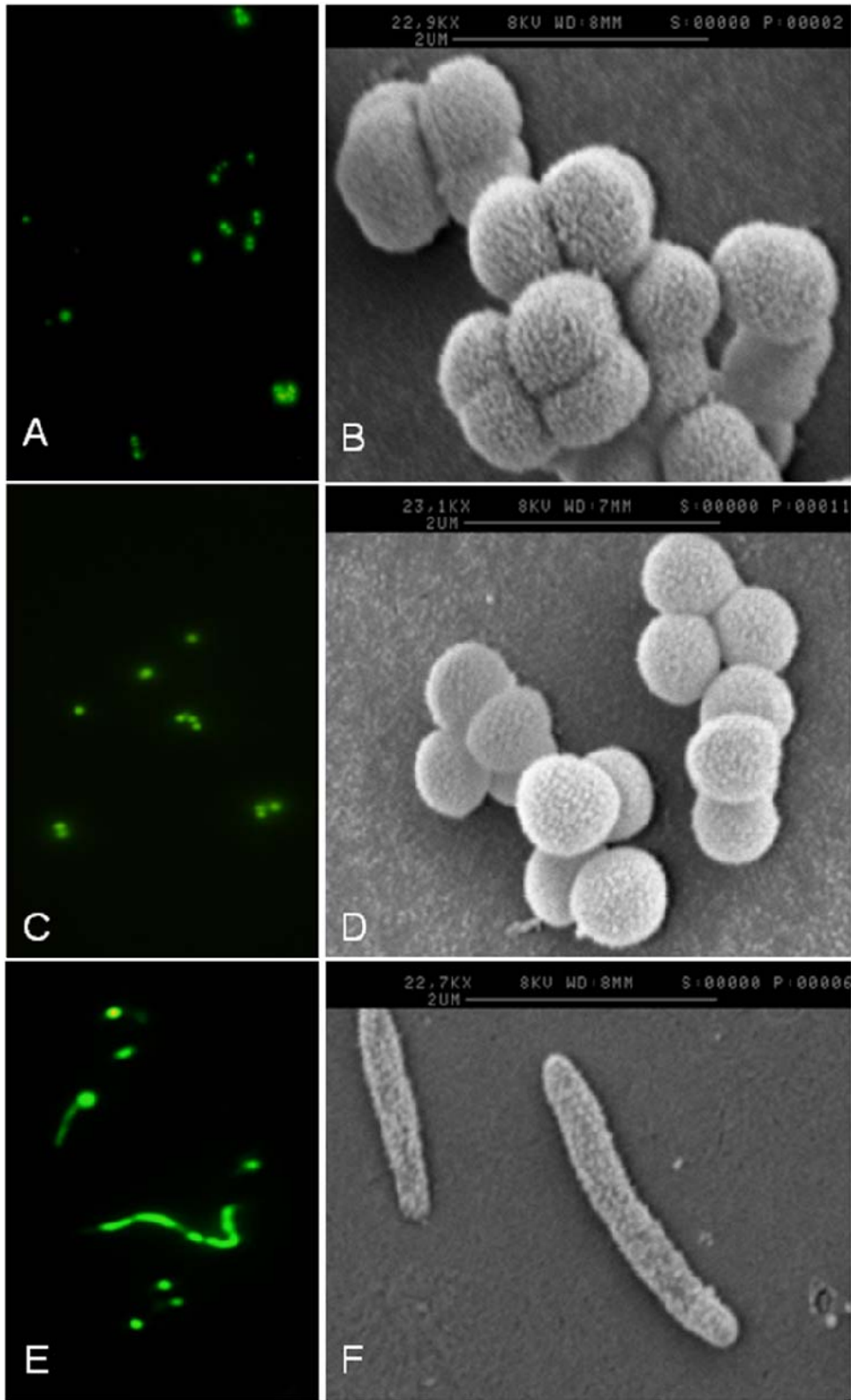


Fig. 3

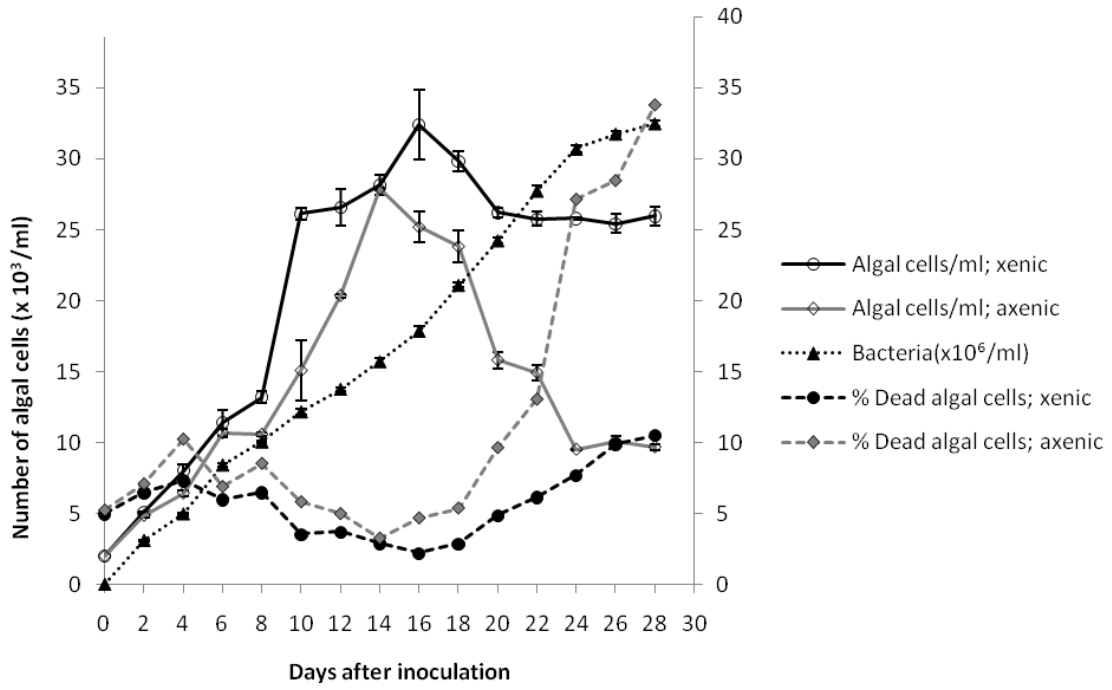


Fig. 4

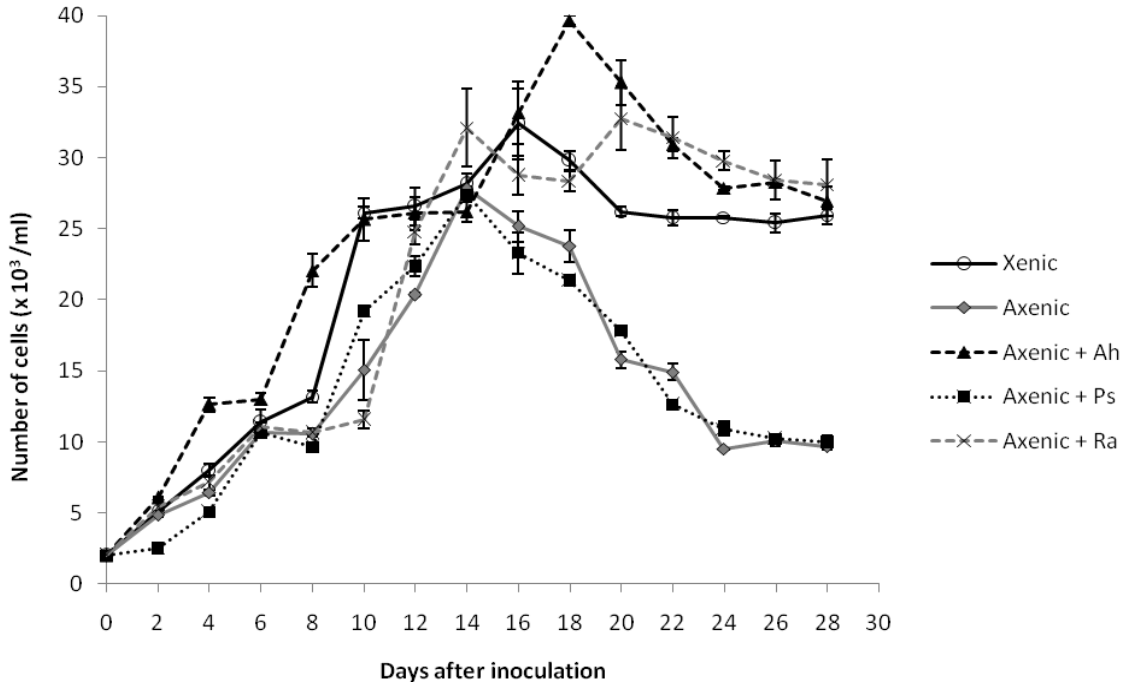


Fig. 5

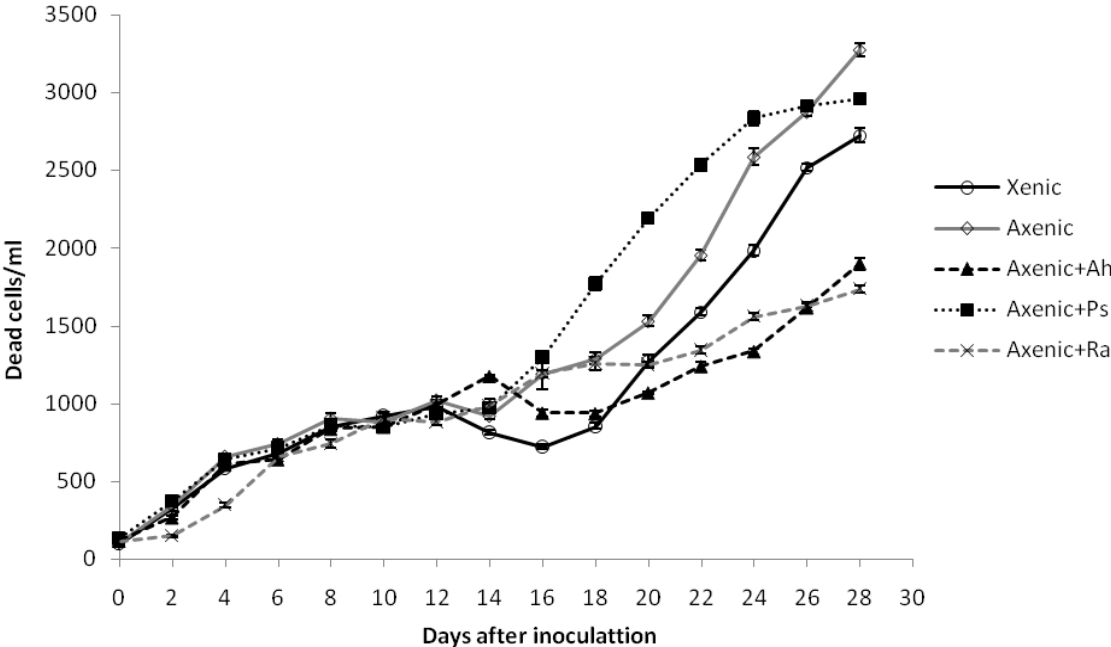


Fig. 6

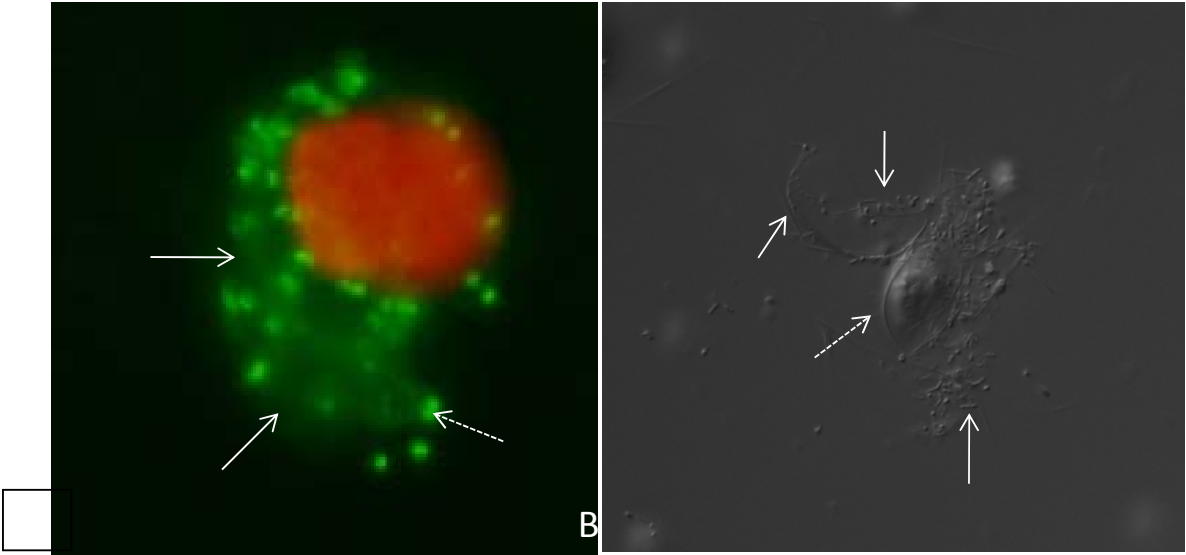


Fig. 7



Fig. 8

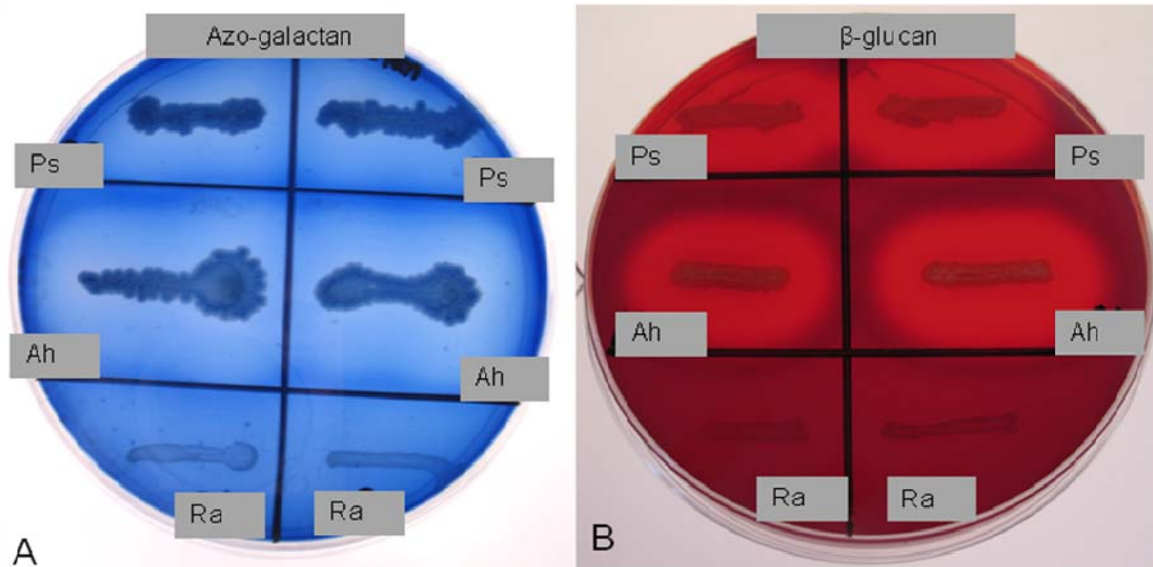


Table 1

Sugar	<i>Pseudomonas</i> sp.	<i>Acinetobacter</i> sp.	<i>Ruegeria</i> sp.
Sucrose	-	+ (with production of gas)	-
Dextrose	+	+	+
Mannose D+	+	+	+
Xylose D+	-	+	+
Maltose D+	-	+	+
Lactose	-	+	-
Mannitol D-	+	+	+
Arabinose L+	+	+	+
Galactose D+	+	+	+
N-acetyl glucosamine	-	+	+
D- glucuronic acid	-	+	+

Table 2

Carbohydrate/Enzyme activity	<i>Pseudomonas</i> sp.	<i>Acinetobacter</i> sp.	<i>Ruegeria</i> sp.
Azo-Galactan (endo-1,4-β-galactanase)	+	+	-
Azo-Carob Galactomannan (β-Mannanase)	+	+	-
β-Glucan (β-glucanase)	+	+	-
Red Pullulan (Dextrinase)	+	-	-
Azo-CM-Cellulose (Cellulase)	-	-	-
Wheat Arabinoxylan	-	-	-
Pectin (Pectinase)	+	+	+
AZ-Rhamnogalacturonan (Rhamnogalacturonanase)	-	-	-