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The effect of high concentration isopropanol on the growth of a solvent-tolerant strain of *Chlorella vulgaris*

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Abstract

The discovery of a highly solvent-tolerant strain of microalga, identified as *Chlorella vulgaris* by 16S rRNA gene sequencing and termed SDC1 is described here for the first time. Ability to grow in the presence of isopropanol (IPA) solvent was assessed through specific growth rate (μ) determination at IPA feed concentrations of up to 16 g l^{-1} at 20 °C. Specific growth rates between 0.0017 and 0.0038 h^{-1} were obtained in the presence of 2–16 g l^{-1} IPA, and a value of 0.0047 h^{-1} evident under IPA-free conditions. Axenic cultures of *C. vulgaris* SDC1 also demonstrated heterotrophic bioconversion of IPA at these elevated concentrations, where acetone, the suggested metabolite, was monitored as an indicator of aerobic degradation of IPA. Comparison of *C. vulgaris* SDC1 growth characteristics was carried out against that of the type culture strain, *C. vulgaris* Beijerinck (CCAP211/11B), where the solvent-tolerant strain SDC1 displayed lower sensitivity to high IPA concentrations. An LD_{50} value was found to occur at initial IPA concentrations of 3.8 g l^{-1} for the type strain in comparison with 11.25 g l^{-1} for the solvent-tolerant SDC1 strain. Despite several studies discussing the existence of solvent-tolerant bacteria, this is the first time solvent-tolerance at such high IPA concentrations (to 16 g l^{-1}) has been successfully demonstrated by an isolated *C. vulgaris* strain in a mineral salts medium.

Keywords: Solvent-tolerant; *Chlorella vulgaris*; Isopropanol (IPA); Microalgae; Bioconversion

1. Introduction

The widespread use of isopropanol (IPA), also known as 2-propanol or isopropyl alcohol, as a precursor or solvent in the manufacture of pharmaceuticals, rubber, fine chemicals and textiles, for example, has resulted in the increased production of organic solvent-containing waste streams [1]. In the UK, for example, isopropanol is the single biggest contributor (6.7%) to total volatile organic compound (VOC) emissions, which are the subject of increasingly stringent legislation [2]. High concentrations of IPA are toxic to most microorganisms due to its high oxygen demand and membrane disruptive characteristics hence its commonplace usage at high concentrations as a disinfectant [3]. Niemi demonstrated that compounds containing isopropyl groups are more resistant to microbial breakdown, suggesting that IPA-containing waste streams may be more problematic than 1-propanol-containing streams within the ecosystem [4]. There are, in fact, fewer reports of IPA-degrading microorganisms than methanol- and ethanol-utilizers. However, certain microorganisms may exhibit solvent-tolerant characteristics [5] although reports of such are mainly limited to bacterial species, with over 70% coming from *Pseudomonas* sp., and little information currently available on this phenomenon in eukaryotes.

Microalgal cultures have received much attention because of their potential in biotechnological applications such as CO_2 fixation and the production of vitamins, pharmaceuticals, fine chemicals and food additives [6,7]. Many of these compounds are synthesized in small quantities and display a low water solubility, which causes significant process development issues for scale-up. Addition of an organic phase to the growth medium may assist with product excretion and product extraction, allowing for enhanced product recovery within the two-phase system [8]. Practically no studies exist regarding freshwater microalgal species, and specifically *Chlorella vulgaris*, in two-phase conditions and the solvent-tolerance effects demonstrated in the presence of C3 alcohols such as 1-propanol or IPA.

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Microalgae also play a role in determining the fate of toxic compounds in the environment and have been shown to accumulate and/or degrade pesticides, aromatic compounds, azo dyes and diesel fractions at concentrations up to 0.2 g l^{-1}, for example [9–12]. Although microalgae utilize light energy and assimilate CO_2, the mechanisms of heterotrophic growth on organic carbon sources are still not fully understood [13]. This is also the case for the biodegradation pathways for many solvents, such as IPA for example, although extensive toxicity studies have been carried out on photoautotrophic unicellular microorganisms such as *Chlamydomonas reinhardtii*, *Anabaena variabilis*, *Monoraphidium braunii* and *Dunaliella salina* in the presence of C5–C8 alcohols and C5–16 alkane solvents [14]. Previous work has also shown that the growth of *Chlorella* VT-1 is reduced in the presence of chlorophenols although no degradation products were detected in the medium [15]. This strain also demonstrated some level of tolerance to a range of chlorophenols, where initial growth was inhibited but the culture subsequently resumed growth at a rate comparable with chlorophenol-free experiments [16].

Here, we describe for the first time the existence of an environmental solvent-tolerant strain of *C. vulgaris*, termed SDC1, and its ability to tolerate high IPA concentrations of up to 16 g l^{-1}. Identification of the *C. vulgaris* SDC1 was carried out by DNA extraction, agarose gel electrophoresis, 16S rRNA PCR amplification, direct DNA product sequencing and sequence analysis as described elsewhere [17]. In addition to survival in the presence of IPA, *C. vulgaris* SDC1 was also capable of converting a proportion of the IPA within a mineral salts medium, and subsequently excreting the main aerobic breakdown product, acetone, into the extracellular medium. Comparison of growth characteristics have also been drawn where μ was determined from the slope of linear semilogarithmic plots of cell number during exponential growth phase for both type and solvent-tolerant strains of *C. vulgaris* in the presence of IPA.

2. Materials and methods

2.1. Isolation of solvent-tolerant microalga

Samples of a bacterial/microalgal mixture were extracted from a contaminated biofilter containing a solvent-tolerant bacterial consortium treating high concentration IPA vapour ($\geq$10,000 ppm_v) at Heriot-Watt University, Edinburgh, UK. Aliquots of the bacterial/microalgal mixture were placed within 250 ml Erlenmeyer flasks containing 100 ml Minimal Salts Medium (MSM) at pH 6 (3 g l^{-1} $NaHCO_3$; 1 g l^{-1} NH_4HCO_3; 0.2 g l^{-1} K_2HPO_4; 102.5 mg l^{-1} $MgSO_4{\cdot}7H_2O$; 36.75 mg l^{-1} $CaCl_2{\cdot}2H_2O$; 10 mg l^{-1} $FeSO_4$; 1 ml l^{-1} Trace Elements Solution), adapted from [18] under natural cyclical daylight conditions. No additional carbon source other than atmospheric CO_2 was added thereby selecting positively for photosynthetic autotrophic microalga. Samples were also grown on Proteose Medium agar slopes (1 g l^{-1} Proteose Peptone; 15 g l^{-1} Agar; 250 mg l^{-1} $NaNO_3$; 175 mg l^{-1} KH_2PO_4; 75 mg l^{-1} $MgSO_4{\cdot}7H_2O$; 75 mg l^{-1} K_2HPO_4; 25 mg l^{-1} $CaCl_4{\cdot}2H_2O$; 25 mg l^{-1} NaCl) to ensure no bacterial contamination. Colonies of *Chlorella* were isolated with an inoculation loop for growth in liquid culture. Reinoculation cycles were carried out over a 2-month period and cells examined on an Olympus CH-2 light microscope to confirm the presence of an axenic *Chlorella* culture.

Chlorella vulgaris Beijerinck (CCAP211/11B) was obtained from the Culture Collection of Algae and Protozoa, Ambleside, Cumbria UK as a comparative type culture strain for solvent-tolerance experiments.

2.2. Cultivation of solvent-tolerant microalgae

An aliquot (1 ml) of an axenic *C. vulgaris* SDC1 cell suspension was taken from the enrichment flasks described above and added to 250 ml flasks containing 8 g l^{-1} IPA in 100 ml MSM. Cultures were statically grown at 20 °C, gently bubbled with air containing 5% (v/v) CO_2 and illuminated with cool white light from fluorescent lights at 150 μE m^{-2} s^{-1} at the surface of the flasks as described by [14] with a 12-h light:12-h dark cycle.

Samples (1 ml) were extracted periodically and cell growth measured. Cell number was calculated using a haemocytometer slide and an Olympus CH-2 light microscope to correlate optical density (OD) values to cell number. The OD was measured on a Hach DR/2000 Spectrophotometer at $\lambda = 582$ nm. Additional microscopic visualization and spread plates were carried out, prior to initiation of each set of experiments, to ensure no microbial contamination.

2.3. Solvent-tolerance experiments

250 ml flasks were set up in at least triplicate containing 2, 4, 8 or 16 g l^{-1} IPA within 100 ml MSM and 1 ml of algal inoculum (1×10^6 cells ml^{-1}) added. Flasks were stoppered with foam bungs and cultivated at light intensity of 150 μE m^{-2} s^{-1} at the surface with 12-h light:12-h dark cycle for the duration of the experiment. In addition, triplicate controls were set up containing the corresponding concentration of IPA to allow correction for non-biological volatilization and photodegradation of solvent. Positive controls comprising 1 ml of either type culture *C. vulgaris* or *C. vulgaris* SDC1 inoculum and MSM without solvent were also included. Triplicate samples of the cell suspensions (1 ml) were taken at regular periods following thorough agitated mixing of reaction media for analysis.

2.4. Solvent concentration determination

Isopropanol (IPA) and acetone concentrations were determined using a Shimadzu GC-17A Gas Chromatograph

equipped with a Carbowax BP20 column (length = 30 m, 1 μm film). Flow rate of He carrier gas was 10 ml min^{-1}, the temperatures of the Flame Ionization Detection (FID) system and oven were 280 and 80 °C, respectively. Injection port temperature was 270 °C and run time was 2 min. Typically, 1 ml of well-mixed cell suspension and medium was extracted from each flask at specified time intervals and centrifuged in an Eppendorf microcentrifuge at 13,000 × *g* for 10 min to pellet the cells. A 1 μl sample of the supernatant was then injected directly into the gas chromatograph for analysis.

2.5. Nucleotide sequence accession number

The nucleotide sequence used for the solvent-tolerant *C. vulgaris* SDC1 culture studied in this work has been deposited with the GenBank database under the accession number AF350260.

3. Results

Growth of *C. vulgaris* SDC1 on IPA feed concentrations of 2–16 g l^{-1} was successfully carried out at a light intensity of 150 μE m^{-2} s^{-1} at the surface with a 12-h light:12-h dark cycle. The typical growth behaviour for *C. vulgaris* SDC1 growing on a minimal salts medium (MSM) in the presence of high concentration IPA within the range 0–16 g l^{-1} is shown in Fig. 1. The addition of IPA to the culture medium reduced the initial growth rate in a concentration-dependent manner, where the IPA-negative control displayed the highest growth rate (0.0047 h^{-1}) and greatest cell density achievable (1.65 units $OD_{582\,nm}$) of all experiments carried out. As the concentration of IPA increased, a considerable decrease in the specific growth rate was evident indicating a solvent inhibition effect. Even at higher IPA concentrations of 16 g l^{-1}, although the lag phase was substantially increased, *C. vulgaris* SDC1 displayed healthier growth after 200 h, demonstrating that the majority of cells had not been irreversibly damaged but were resistant to the solvent effects. Fig. 1 also demonstrates that the final cell density obtainable is reduced in the presence of increasing IPA concentrations. Between initial IPA concentrations of 2–8 g l^{-1}, cell densities are reduced by approximately 10% for every additional 2 g l^{-1} IPA added to the culture medium, while a biomass reduction of only 40% was evident for *C. vulgaris* SDC1 in the presence of 16 g l^{-1} IPA, indicating a non-linear trend for biomass concentration reduction against increased solvent concentration.

In order to assess whether the solvent-tolerant characteristics where adaptive or inherent in other strains of *C. vulgaris*, the specific growth rates of SDC1 and the type collection strain, *Chlorella vulgaris* Beijerinck (CCAP211/11B), were examined in parallel utilizing the same range of IPA

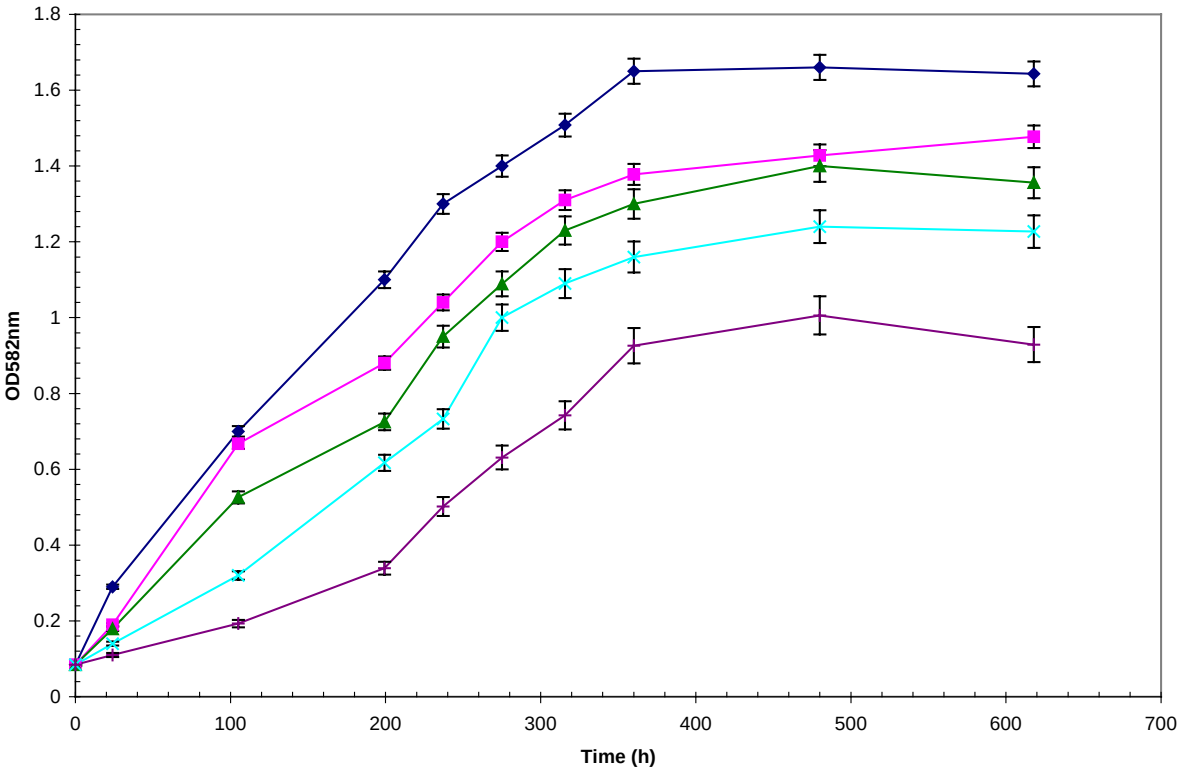

Fig. 1. Growth profiles of *C. vulgaris* SDC1 in a minimal salts medium in the presence of IPA. Initial IPA concentrations of 2 (■), 4 (▲), 8 (×) and 16 (+) g l^{-1} were examined in addition to IPA-negative control samples (◆).

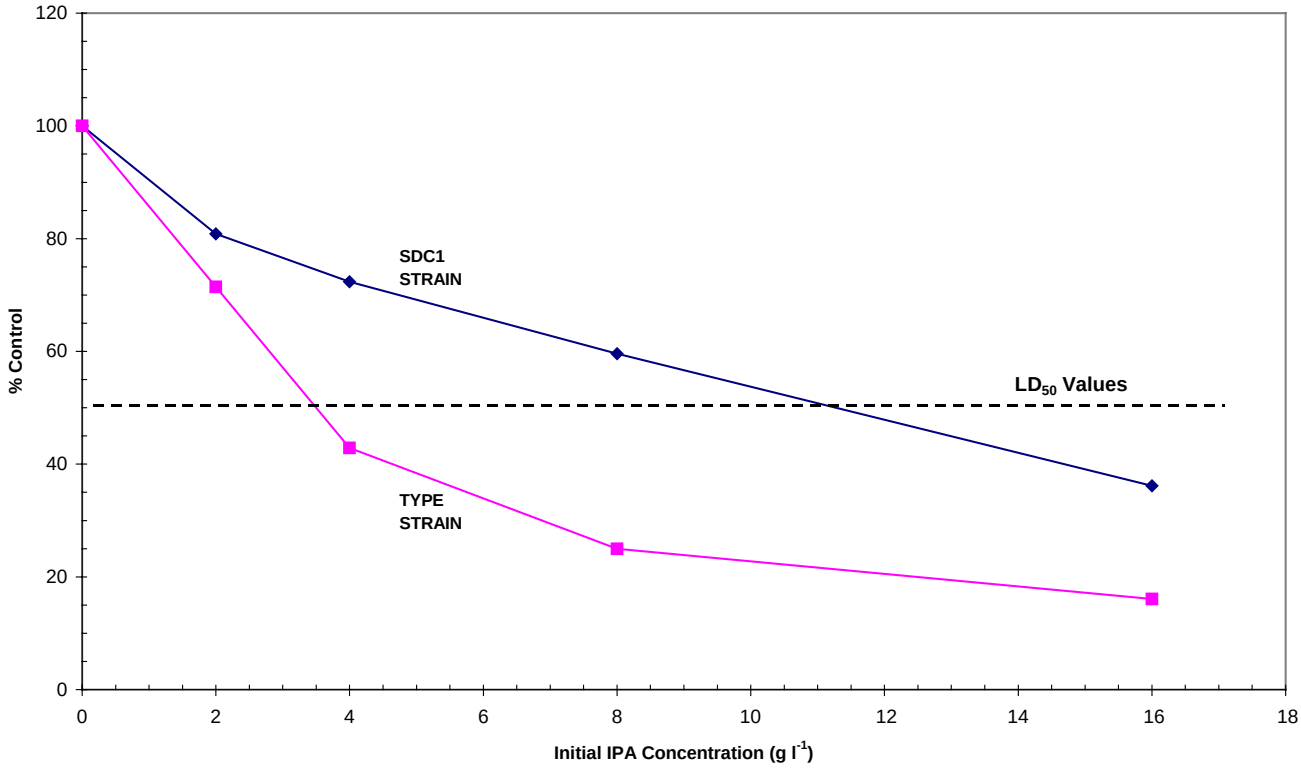

Fig. 2. Specific growth rates of *C. vulgaris* SDC1 (◆) and type strain *C. vulgaris* Beijerinck (CCAP211/11B) (■) in the presence of a range of IPA concentrations to 16 g l^{-1} in a minimal salts medium. The LD_{50} values have also been indicated (- - -) for both strains.

concentrations. Specific growth rates were calculated by taking the slope of the exponential phase of growth of a semi logarithmic plot of cell density versus time. It is clear from Fig. 2 that the SDC1 environmental strain was generally less sensitive to higher concentrations of IPA in the growth medium, whereas the type strain exhibited a 29% reduction in specific growth rate in the presence of only 2 g l^{-1}. A similar reduction in SDC1 growth rate was evident, but only in the presence of 4 g l^{-1} IPA thus indicating a greater degree of solvent-tolerance. The largest differential in growth rate reduction between the type strain and SDC1, in comparison to their respective controls, occurred in the presence of 8 g l^{-1} IPA, where a 35% difference was apparent. The LD_{50} values (50% lethal dose) have been determined from Fig. 2 using the percentage reduction of the IPA-negative control growth rate for SDC1 and the type strain *C. vulgaris* Beijerinck (CCAP211/11B). The LD_{50} for SDC1 was demonstrated to be 11.4 g l^{-1} whereas the type *C. vulgaris* strain displayed an LD_{50} value of just 3.8 g l^{-1}.

Throughout the course of the solvent-tolerance experiments IPA bioconversion by *C. vulgaris* SDC1 was observed that was not due to volatilization or photodegradation since evaporation controls had been utilized. Fig. 3A depicts the volatilization-corrected removal of IPA from the growth medium for the concentration range 2–16 g l^{-1}. Total IPA removal occurred after 450 h cultivation for a 2 g l^{-1} feed whereas both the 4 and 8 g l^{-1} solvent was removed following 625 h growth. The 16 g l^{-1} IPA feed was not completely removed from the culture medium during the time course of the experiment but reached a residual value of 6.4 g l^{-1} after 625 h cultivation. The minor decrease in IPA concentration following inoculation may be due purely to absorption phenomenon whereby the cells are unable to control the intracellular passage of the solvent since there is no measurable bioconversion byproduct.

Acetone, which is the main indicator of aerobic mineralization of IPA in other biological systems, was also found to accumulate in the *C. vulgaris* SDC1 culture medium. Extracellular acetone was detected as a byproduct of aerobic IPA bioconversion during growth of SDC1 in the presence of IPA and this is shown in Fig. 3B. No acetone was detectable in the growth medium when SDC1 was grown in the absence of IPA or when cell-free evaporative controls were monitored. Throughout all IPA feed concentrations studied, it is clear that lower concentrations of IPA yield highest extracellular quantities of acetone. The maximum acetone concentration generated was 0.54 g l^{-1} at an initial IPA feed of 2 g l^{-1} with the trend following a concentration-driven decrease in extracellular acetone to 0.48 and 0.36 g l^{-1} for initial IPA concentrations of 4 and 8 g l^{-1}, respectively. There appears to be an increasingly evident two-rate trend for evolution of acetone with an increase in IPA feed, as typified by SDC1 growth in the presence of 16 g l^{-1} where a clear shift in acetone generation was demonstrated following 210 h cultivation. This

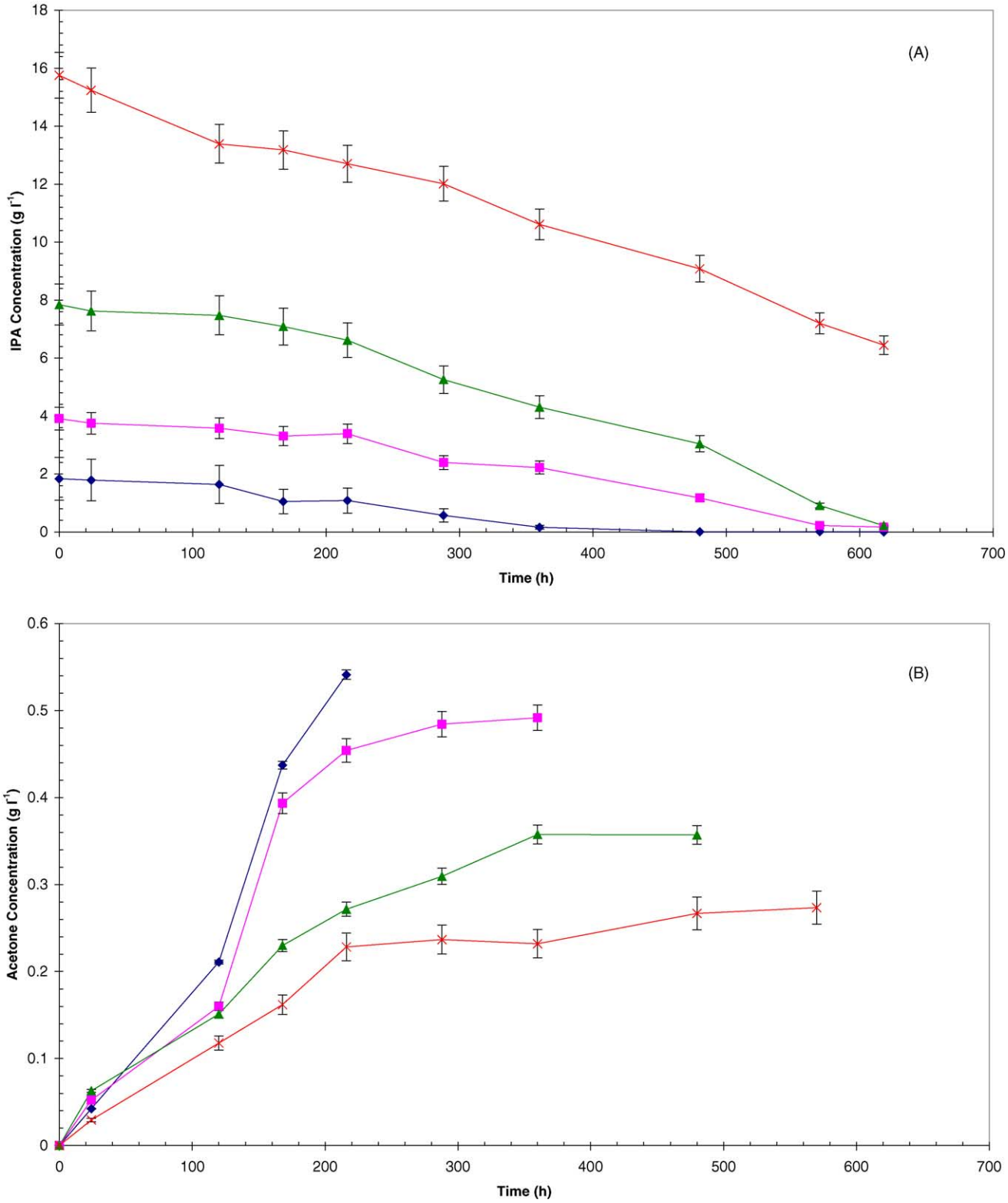

Fig. 3. (A) IPA concentration profile during cultivation of *C. vulgaris* SDC1 in a minimal salts medium containing IPA concentrations of 2 g l^{-1} (◆), 4 g l^{-1} (■), 8 g l^{-1} (▲) and 16 g l^{-1} (×). All IPA samples have been corrected for volatilization and photodegradation via cell-free controls. (B) Acetone generation into the medium during growth of *C. vulgaris* SDC1 at initial IPA concentrations of 2 g l^{-1} (◆), 4 g l^{-1} (■), 8 g l^{-1} (▲) 16 g l^{-1} (×). All acetone samples were corrected for volatilization and photodegradation via cell-free controls. No acetone was detectable when *C. vulgaris* SDC1 was grown in IPA-negative controls.

shift in rate of acetone generation was not purely due to the switch from exponential to stationary during cultivation and it seems likely that alteration in metabolic activity may have occurred.

4. Discussion

This work represents, not only the first demonstration of the growth characteristics of the solvent-tolerant *C. vulgaris* SDC1 in the presence of high concentrations of IPA, but also its first comparison with a type *C. vulgaris* strain. The growth of *C. vulgaris* SDC1 was successfully achieved in a minimal salts medium rather than Bolds Basic, Sorokin and Krauss Media in the presence of up to $16\,g\,l^{-1}$ IPA. The strain showed a more obvious biphasic growth pattern with increasing IPA feed concentrations but which was probably not due to shading and carbon dioxide limitation, which are common when the culture density increases [19]. An increase in IPA feed concentration inhibited the growth of both the environmental and type strains of *C. vulgaris* although the type strain was more sensitive to the presence of even low concentrations of IPA than the solvent-tolerant SDC1 strain, whose growth appeared more robust. This is clear from the lethal dose values calculated for both strains, with SDC1 exhibiting an LD_{50} more than three-fold higher than the type strain and hence indicating its inherent solvent-tolerance. Since this strain was isolated from a gas-phase biofilter treating high-concentration solvent wastestreams, it is comprehensible that the long-term selection process has insured that cells survive and grow in what would otherwise be a toxic environment [20]. This approach may also yield more robust strains demonstrating improved degradation of a range of toxins over time within increasingly extreme environments not usually capable of supporting significant microbial growth.

Previous studies examining the toxicity effects of solvents (to $4.6\,g\,l^{-1}$) such as ethanol, methanol, dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF) have demonstrated a significant reduction in specific growth of *C. vulgaris* and *Selenastrum capricornutum* green algae [21]. However, here we demonstrate the growth characteristics of *C. vulgaris* SDC1 in IPA concentrations of up to $16\,g\,l^{-1}$ and additionally elucidate that this strain is capable of bioconversion of IPA to acetone. There appears to be no clear trend regarding the link between the presence of acetone in the culture medium and initial IPA concentration, since maximum acetone generation does not always appear to coincide with the transition from exponential growth into the stationary phase. Although a range of solvents have been examined for their toxicity effects on both marine and freshwater microalgal species, information is sparse regarding IPA and acetone tolerance. In other algal species very little information is available on either alcohol- or acetone-converting enzymes, although Grondal et al. have partially purified an alcohol dehydrogenase from the unicellular green alga *Chlamydomonas moewusii* [22].

To date, over 70% of solvent-tolerant microbial strains isolated belong to the genus *Pseudomonas* [23]. However, here we report for the first time the existence of the eukaryotic solvent-tolerant *C. vulgaris* SDC1 capable of growth in IPA at concentrations of up to $16\,g\,l^{-1}$ and capable of superior growth in the presence of solvent than its type collection counterpart. It has also been demonstrated that not only does this *C. vulgaris* survive at these concentrations, but also that it is capable of some degree of IPA bioconversion at up to $16\,g\,l^{-1}$. Assumptions have been made that solvent-tolerance may be due to a combination of mechanisms such as solvent-tolerant enzyme utilization, membrane structure modification, membrane pump activity and diffusion in both bacterial and algal cells [24]. It is still not categorically concluded whether this solvent-tolerance is inherent in all *Chlorella* species or whether it is an effect brought about by long-term environmental selection, as suggested for this SDC1 strain. However, initial comparisons with a type *C. vulgaris* strain infer that it may be somewhat inherent but that environmental selection causes considerable enhancement. In the future, the exploitation of such solvent-tolerant photosynthetic microalgae offers several options in the field of environmental biotechnology and biocatalysis at both micro- and large-scale operation.

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