Biodegradation of high-concentration isopropanol by a solvent-tolerant thermophile, *Bacillus pallidus*

Mark T. Bustard, Samantha Whiting, Don A. Cowan, Phillip C. Wright

Abstract
The aerobic biodegradation of high-concentration, to 24 g l^{-1}, 2-propanol (IPA) by a thermophilic isolate ST3, identified as *Bacillus pallidus,* was successfully carried out for the first time. This solvent-tolerant *B. pallidus* utilized IPA as the sole carbon source within a minimal salts medium. Cultivation was carried out in 100-ml shake flasks at 60° C and compared with cultivation within a 1-l stirred tank reactor (STR). Specific growth rate (μ) was about 0.2 h^{-1} for both systems, with a maximum cell density of 2.4 x 10^8 cells ml^{-1} obtained with STR cultivation. During exponential growth and stationary phase, IPA biodegradation rates were found to be 0.14 and 0.02 g l^{-1} h^{-1}, respectively, in shake-flask experiments, whereas corresponding values of 0.09 and 0.018 g l^{-1} h^{-1} were achievable in the STR. Generation of acetone, the major intermediate in aerobic IPA biodegradation, was also monitored as an indicator of microbial IPA utilization. Acetone levels reached a maximum of 2.2-2.3 g l^{-1} after 72 and 58 h for 100-ml and 1-l systems, respectively. Both IPA and acetone were completely removed from the medium following 160 and 175 h, respectively, during STR growth, although this was not demonstrated within shake-flask reactions. Growth of *B. pallidus* on acetone or IPA alone demonstrated that the maximum growth rate (μ) obtainable was 0.247 h^{-1} at 4 g l^{-1} acetone and 0.202 h^{-1} at 8 g l^{-1} IPA within shake-flask cultivation. These results indicate the potential of the solvent-tolerant thermophile *B. pallidus* ST3 in the bioremediation of hot solvent-containing industrial waste streams.

Key words *Bacillus pallidus*; Biodegradation; Isopro-panol (IPA); Solvent tolerant; Thermophilic; Volatile organic compounds (VOCs)

Introduction

Volatile organic compound (VOC) emissions are becoming increasingly more regulated, thus forcing technologists to assess more efficient and economic process options (Kiared et al. 1997). The most widely used VOC is 2-propanol, or isopropyl alcohol (IPA), whose production worldwide exceeds 1 million tonnes per annum, through its many industrial uses as a cheap ethanol replacement for solvent, intermediate, de-icer, and applications in rubber, cosmetics, textiles, pharmaceuticals, and fine chemicals industries (Harris 1991; Derwent and Pearson 1997).

Biological treatment of IPA-containing waste streams can be advantageous (Lobos et al. 1992), but little information is available on this process at high temperature and high solvent concentration, despite several successful meso-philic anaerobic studies involving co-metabolism with glucose (Fox and Ketha 1996; Ueyama et al. 1971). Although IPA and toluene are typical solvents conveniently used for sterilization,

some microorganisms may assimilate these toxic compounds at low concentrations (Kanemitu et al. 1980; Murrays et al. 1980). This process is described in more detail elsewhere (Bustard et al. 2000).

In order to develop both conventional and novel bio-reactors, substrate transport phenomena, biodegradation rates, and biomass growth must be mechanistically-scaled (Dolfing and Janssen 1994; Fujita et al. 1992; Singleton 1994). Quantification of bioconversion kinetics is essential in waste gas biofiltration or wastewater treatment system design, with both single- and multi-substrate biodegrada-tion applications demonstrated (Guha et al. 1999).

Previous studies involving *Bacillus pallidus* strains have examined the characterization of their thermostable nitrile hydratase and nitrilase enzymes (Cramp and Cowan 1999; Almatawah and Cowan 1999). However, the potential of *B.pallidus* in pollution abatement systems has been generally overlooked, with only hot composting, pinene (to $2gl^{-1}$), and phenol (to 3 g l^{-1}) degradation applications mentioned in the current literature (Ali et al. 1998; Savithiry et al. 1998; Blanc et al. 1997). By and large, thermophilic bacteria are often applied in waste stream treatment systems, such as gas biofiltration and anaerobic digesters (Dhamwichukorn et al. 2001; Han and Dague 1997). Here we describe, for the first time, the aerobic thermophilic biodegradation of high concentrations of IPA by a *B. pallidus* isolate, focusing on specific growth rate, IPA biodegradation, and acetone generation. The scale-up fermentation of this thermophile utilizing high-concentration IPA as the sole carbon source, the effect on cell growth, and the effect on IPA degradation rate are also described.

Materials and methods

Isolation of microorganism

The thermophilic strain was isolated from a phenol-contaminated industrial effluent lake (Huntly, New Zealand), and identified as described previously by Ali et al. (1998). Single colony-forming units were selected from nutrient agar plates (13 g l^{-1} nutrient broth, 1.2% (w/v) bacteriological agar; Difco) inoculated with this IPA-utilizing *B. pallidus* strain. Subsequently, this pure strain was cultivated at 60° C ($T_{optimum}$ - data not shown) in submerged culture, and examined under an Olympus CH-2 light microscope to ensure single strain purity. This was carried out both prior to inoculation and following conclusion of the reaction.

Enrichment procedure

The *B. pallidus* ST3 isolate was initially cultivated in $13gl^{-1}$ nutrient broth (Oxoid, UK) in a lateral shaking water bath set at 150 rpm and 60° C. All media was auto-claved at 121° C, 1 atm for 15min and allowed to cool to 60°C prior to inoculation. Cycles of growth were carried out with 1% (v/v) inoculum added to 100 ml of 13 g l^{-1} nutrient broth (Difco) in 250-ml Erlenmeyer flasks at 60° C. which were then converted to cell number (cells ml^{-1}) using a calibration curve. Controls containing MSM and either $2\text{-}24gl^{-1}$ IPA or acetone were set up to correct for volatilization and evaporative loss during the course of the experiment. These volatilization controls then allowed true bacterial biodegradation rate to be determined rather than combined volatilization/biodegradation. These true values are depicted in the Figures.

Solvent concentration determination

IPA and acetone concentrations were determined using a Shimadzu GC-17A gas chromatograph equipped with a Carbowax BP20 capillary column (length 30 m; i.d. 0.53 mm; 1 \i m film). Flow rate of He carrier gas was 11 ml min^{-1}, the temperatures of the flame ionization detection (FID) system and injection port were 280° C. The oven temperature was 80°C and sample run time was 2 min. Typically, 1 ml of culture was extracted from each reaction at specified time intervals, cells were spun down in an Eppendorf benchtop microcentrifuge at 12,000 xg, and a l sample of supernatant injected into the gas chromato-graph for analysis. Samples were extracted and analyzed in triplicate.

Batch fermentation scale-up

An autoclaved 1-l stirred tank reactor (STR) containing 800 ml MSM was inoculated with 10 ml of culture (1.4 x 10^7 cells ml^{-1}). Filtered (0.2 um) air was supplied at a rate of 0.02 l min^{-1} and the reaction mixture stirred by an impeller and overhead motor (OKI) at 150 rpm. The STR temperature was maintained at 60°C by means of a water jacket, and gas vented via a 0.45-um filter to maintain sterility. Biodeg-radation of IPA was carried out through batch addition of 8.8 ml IPA solvent to give a final concentration of 8 g l^{-1} in the reaction vessel. Identical control reactors were set up simultaneously with IPA or acetone alone, under the same conditions but without *B. pallidus* inoculum, to determine volatilization and evaporative loss rate under reaction conditions. These evaporation rates were then subtracted from combined volatilization/biodegradation rates to obtain true microbial biodegradation rates as depicted in the Figures. Experiments were carried out three times.

Solvent degradation experiments

Several 250-ml shake flasks were set up in at least triplicate containing 0-24 g l^{-1} IPA or acetone within a 100-ml minimal salts medium (MSM), as described by Bustard et al. (2000), and 1ml of inoculum (1.4 x 10^7 cells ml^{-1}) added. IPA or acetone was added as the sole carbon nutrient source. Flasks were stoppered with foam bungs, covered in aluminum foil, and placed within a variable-temperature shaking incubator at 60°C for the duration of the experiment. Samples were taken periodically for cell number and solvent concentration analysis. Cell growth was measured using OD_{450} values, as described by Bustard et al. (2000),
Nucleotide sequence accession number

The partial 16S rDNA gene sequence obtained for *B. pallidus* ST3, utilizing methodology described by Rainey et al. (1994), has been deposited in the GenBank database under accession number AF380929.

Results

After numerous serial transfers during a 6-month period, a bacterial strain, designated ST3, capable of growth in the presence of 8 g l^{-1} IPA was isolated. Partial 16S rDNA sequencing over 600 bp revealed 99%

homology to *B. pallidus* and displayed a close relationship to other thermophilic *Bacillus* spp. (Ali et al. 1998; Rainey et al. 1994).

This *B. pallidus* isolate demonstrated the ability to grow on high-concentration IPA as the sole carbon source when grown in shake-flask culture at 60° C. Figure 1 (after References) shows that exponential phase growth is initiated following a 23-h lag phase. The specific growth rate of *B. pallidus* on IPA was calculated to be 0.202 h^{-1} by taking the slope of the exponential growth phase. It was also demonstrated that a maximum cell density of 1.8 x 10^8 ml^{-1} was achievable during shake-flask cultivation on 8 g l^{-1} IPA solvent. During cell growth in exponential phase through to stationary phase, a maximum IPA biodegradation rate of 0.141 g l^{-1} h^{-1} was exhibited. However, the rate of IPA utilization during stationary phase (steady state) was reduced to 0.021 g l^{-1} h^{-1}, more than six times lower than that demonstrated during the exponential phase.

The presence of acetone, the major intermediate of aerobic IPA biodegradation (Steffan et al. 1997), was also monitored throughout the experiment, and was found to appear after 24 h of cultivation (Fig. 1). Acetone reached a maximum level of 2.2 g l^{-1} following 72 h of reaction, and was then found to be utilized as a substrate itself by *B. pallidus*, showing a concentration reduction during the stationary phase of cell growth between 72 and 144 h. Cell death was initiated at 154 h, with final IPA and acetone concentrations of 0.32 and 1.15 gl^{-1} present, respectively. All biodegradation experiments were corrected for IPA and acetone volatilization and evaporative loss.

A comparison of *B. pallidus* specific growth rates, μ (h^{-1}), for acetone and IPA alone over the concentration range 0-24 gl^{-1} solvent in shake-flask cultivation experiments is depicted in Fig. 2 (after References). At initial solvent concentrations to 8 g l^{-1}, more rapid cell growth is observed when acetone is the sole carbon source, with m values of up to 0.247 h^{-1} obtainable, whereas *B. pallidus* growth on IPA is maximal at an initial concentration of 8 g l^{-1}. At solvent concentrations of 2 and 4gl^{-1}, μ values of almost four- and two-fold higher, respectively, for acetone over IPA are demonstrated. Acetone is clearly observed to be the preferred substrate over IPA at low initial solvent concentrations, although this was not the case at significantly higher solvent concentrations (to 24gl^{-1}). At these substrate conditions, IPA displayed higher μ values of 0.071 and 0.011 h^{-1} at 16 and 24 g l^{-1} IPA, respectively, in comparison with reduced values of 0.04 and 0.005 h^{-1} for acetone at similar concentrations. This indicates that high concentrations of acetone as the sole carbon source have a greater inhibitory effect on *B. pallidus* growth than similar concentrations of IPA.

In order to ensure more effective control during the *B. pallidus* fermentation, cultivation was carried out within a 1-l STR at 8 g l^{-1} IPA as the sole carbon source, since the highest μ values had been obtained in previous shake-flask cultivation. Aeration and agitation parameters were optimized (data not shown) to ensure high-efficiency bio-degradation of IPA and acetone by *B. pallidus*. It was demonstrated that higher cell biomass was achievable under these conditions (Fig. 3), where 2.4 x 10^8 ml^{-1} was obtained (a 33% increase over the shake-flask growth). The specific growth rate of *B. pallidus* in 1-l batch STR culture was 0.199 h^{-1}, very similar to that obtained for 100-ml shake-flask cultivation (0.202 h^{-1}). However, the IPA biodegradation rates were significantly reduced (by 30%) during the scale-up reaction, where the rate calculated during exponential phase of growth was 0.099 g l^{-1} h^{-1} as opposed to 0.141 g l^{-1} h^{-1} at 100-ml volume. Furthermore, IPA biodeg-radation by *B. pallidus* proceeded at 0.018 g l^{-1} h^{-1} during stationary growth phase (steady state), a conversion reduction of 3 x 10^{-3} gl^{-1} h^{-1} for IPA. This is especially interesting

given that there was a 30% greater biomass concentration in the 1-l STR. Further studies into cell damage and viability are currently under way within our laboratory in an attempt to form a mechanistic understanding of this problem.

Acetone was found to be produced after 26 h, reaching a maximum level of 2.3 g l^{-1} following 58 h of reaction (Fig. 3, after References). The maximum acetone level was achieved 14 h earlier during cultivation within the 1-l STR and this substrate was itself utilized to completion at 175 h. All IPA was effectively removed from the medium following a cultivation time of 160 h, and *B. pallidus* stationary phase was achievable between 70 and 170 h without the onset of cell death. However, although the scale-up reaction was successfully carried out at 1 l, the lower IPA biodegradation rate observed during exponential growth was 0.042 g l^{-1} h^{-1} less than that experienced with 100-ml cultivation. Comparison of Figs. 1 and 3 demonstrates that the onset of stationary phase during 1-l cultivation occurs at 70 h as opposed to 60 h, as observed in smaller-scale cultivation.

Discussion

Cultivation of the thermophile *B. pallidus* was successfully achieved under aerobic conditions using IPA or acetone as the sole carbon source at 60° C. This strain exhibited a degree of solvent tolerance, since solvent was present in the medium at concentrations up to 24 g l^{-1}, where cell densities above 10^8 ml^{-1} were demonstrated. No previous reports of IPA tolerance by thermophilic *Bacilli* are currently available in the literature, although *Bacillus* DS-994, isolated from deep-sea sediment samples, has demonstrated growth in the presence of 5% (v/v) benzene and 10% (v/v) toluene (Moriya and Horikoshi 1993). Here, we demonstrate not only cellular growth in the presence of high-concentration IPA and acetone, but also their utilization as the sole carbon source by *B. pallidus*. Comparison of specific growth rates for *B. pallidus* on acetone or IPA alone showed that acetone was a preferred substrate at concentrations to $8gl^{-1}$, although above this it exhibited greater inhibitory affects on μ values, indicating higher toxicity than IPA above 16 g l^{-1}. Furthermore, order of magnitude greater cultivation reactions were successfully applied in order to optimize the biodegradation of 8 g l^{-1} IPA within a 1-l STR. However, although aeration and mass transfer are increased within a stirred tank reactor, shear forces may also have a detrimental effect on cell growth. Althouth the achievable cell density was increased significantly during scale-up, a later onset of stationary growth phase was demonstrated within the STR than that observed for shake-flask cultures. This may be due to shear stress caused by the mechanical impeller within the vessel, since the aeration rate was sufficient to ensure that oxygen limitation did not occur. This increased aeration also seems to ensure a prolonged stationary period prior to cell death, which benefits the biodegradation reaction sufficiently to ensure that both IPA and acetone are completely utilized. The biodegradation rate has implications with regard to residence time and metabolite production for bioreactor operation in pollution abatement systems such as wastewater treatment or gas biofiltration. In addition, the ability of these solvent-tolerant bacteria to withstand higher fluctuations in pollutant levels, at the elevated temperatures often found industrially, should enable more efficient operation of such systems. Steffan et al. (1997) also suggest that IPA is converted to acetone, which in turn is converted to pyruvic acid and enters directly into the TCA cycle. Although Fox and Ketha (1996) demonstrated the microbial biodegradation of IPA under anaerobic conditions at 30°C, the highest conversion values were through co-metabolism of 1.5 g l^{-1} IPA with 1 g l^{-1} glucose. Here, we demonstrate for the first time the ability of the aerobic thermophile *B. pallidus* to utilize

high concentrations of IPA and acetone at 60°C, with no further additional carbon supplementation under conditions that may be considered solvent-tolerant.

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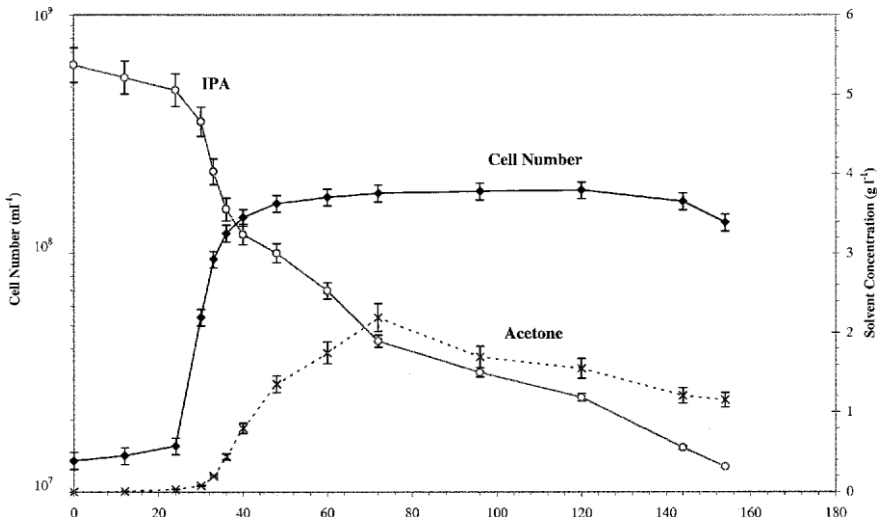

Fig. 1. Biodegradation of 8 g l^{-1} isopropyl alcohol (IPA) by the solvent-tolerant thermophile *Bacillus pallidus* within 100-ml reaction volume at 60° C. IPA concentration *(circles),* acetone concentration (crosses), and cell growth *(diamonds)* are depicted. All experiments were carried out in triplicate, and corrected for volatilization and evaporative loss for both IPA and acetone

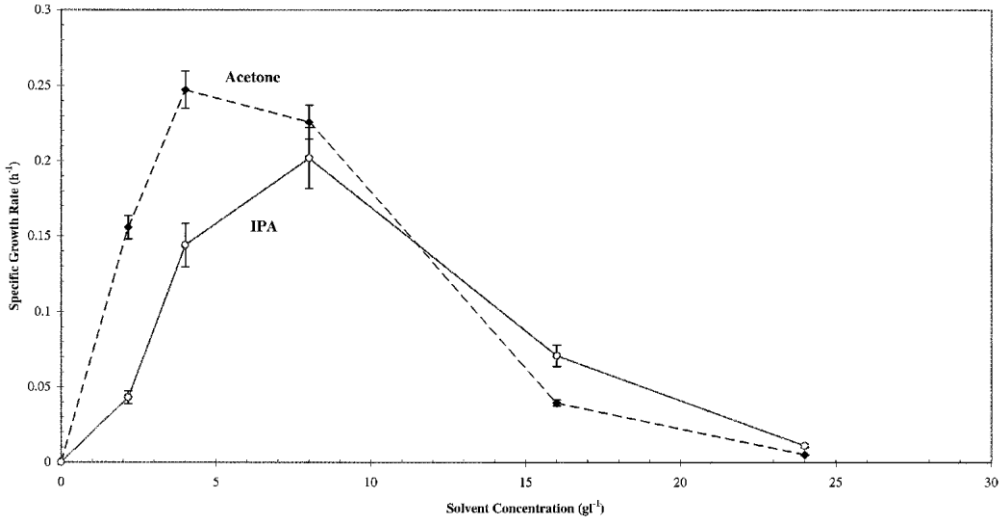

Fig. 2. Specific growth rate, |i (h^{-1}), of *B. pallidus* as a function of initial solvent concentration (g l^{-1}) for either acetone *(diamonds)* or IPA *(circles)*. All experiments were carried out in triplicate shake flasks as detailed previously, and corrected for volatilization and evaporative loss for both IPA and acetone solvents

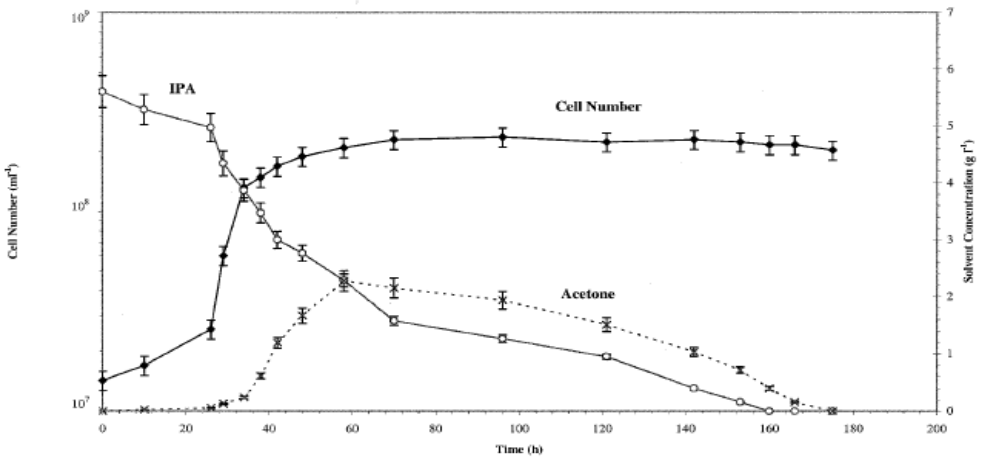

Fig. 3. Cultivation of the solvent-tolerant *B. pallidus* within a 1-l stirred tank reactor (STR) at 60°C using 8 g l–1 IPA as the sole carbon source. IPA concentration (*circles*), acetone concentration (*crosses*), and cell growth (*diamonds*) are shown. All cultivation experiments were carried out in triplicate, and corrected for IPA and acetone volatilization and evaporative loss