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Original Article

Viable but nonculturable state of *Escherichia coli*, *Pseudomonas putida* and *Lactococcus lactis* during exposure to toxic chemicals, as revealed by headspace gas chromatography and indirect conductivity techniques

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Abstract

The bioconversion of water-immiscible chemicals by microbes may occur through viable but nonculturable cells (VBNC) and the evaluation of such cellular activity is important for a deeper understanding of the metabolic process. In this study, the metabolic CO_2 production in a bacteria interface emulsion, consisting of bacteria, chitosan and solvent, has been monitored using headspace gas chromatography (GC) and indirect conductivity (IC). The results from GC in comparison to the standard culture technique revealed the presence of VBNC state when *E. coli* DH5α and *P. putida* F1 were in contact with *n*-hexane. *L. lactis* IO-1 was the most sensitive strain, but the VBNC cells were obvious in the case of soybean and *n*-decane. Although GC showed better detection sensitivity, the IC technique was more practical and cost-effective. Therefore, GC and IC could be extremely simple and useful methods for monitoring the VBNC state of bacteria, which generally stems from environmental stresses.

Keywords: gas chromatography, indirect conductivity, metabolic activity, viable but nonculturable (VBNC), emulsion

1. Introduction

Are the bacterial cells already dead or still alive? This question is definitely important on the basis of decisions when people are concerned about the function of metabolically active bacteria, especially in the bioconversion of toxic water-immiscible chemicals when the cells are utilized as micro-scale factories producing value-added products in contact with organic media (Aono, Tsukagoshi, & Miyamoto, 2001; de Carvalho, & da Fonseca, 2004; Lee, Yun, Lee, & Park, 2015; León, Fernandes, Pinheiro, & Cabral, 1998; Siriphongphaew *et al*., 2012; Wangrangsimagul *et al*., 2012). The common plate count is a traditional method for the enumeration of culturable bacteria, based on the ability to grow and produce colonies on agar media. However, many recent studies have revealed that both Gram-negative and Gram-positive bacteria can enter a viable but nonculturable (VBNC) state, meaning that the bacteria are alive and show metabolic activity and respiration, but cannot be seen as colony forming units on the agar media (Capozzi *et al*., 2016; Kell, Kaprelyants, Weichart, Harwood, & Barer, 1998; Rizzotti, Levav, Fracchetti, Felis, & Torriani, 2015; Wu, Liang, & Kan, 2016; Zhao *et al*., 2017). Therefore, knowing the real status of the bacteria is crucial as regards biosafety concerns as well as utilization of the bacteria as biocatalysts in bioconversion.

In combination with conventional plate counts, several cell viability assessment techniques have been employed to estimate the VBNC cells. These include SYBR green real-time PCR (Tirapattanun, Chomvarin, Wongboot, &

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Kanoktippornchai, 2015), propidium monoazide combined with qPCR (Rizzotti *et al*., 2015; Wu *et al*, 2016) and fluorescein diacetate staining/flow cytometry (Capozzi *et al*., 2016). CO_2 can be a decent candidate for the quantification of viable cells. Headspace gas chromatography (GC) has been used to monitor metabolic CO_2 for estimating bacterial cell numbers in food systems (Guerzoni, Gardini, Cavazza, & Piva, 1987; Gardini, Lanciotti, Sinigagli, & Guerzoni, 1997) as well as in culture media (Chai, Dong, & Deng, 2008). Alternatively, the level of CO_2 can be indirectly measured by trapping CO_2 in an alkaline solution placed in the same vessel with the bacteria, and monitoring conductivity of the alkaline solution; this is referred to as Indirect Conductivity (IC) (Bolton, 1990; Taok, Cochet, Pauss, & Schoefs, 2007; Timms, Colquhoun, & Fricker, 1996). Such system is feasible because the conductivity of hydroxide ions (OH^-) is completely different from that of carbonate ions (CO_3^{2-}). Despite their simplicity and availability, neither GC nor IC technique has yet been reported to assess the VBNC state of bacteria. In this study, a commercial portable conductivity probe was for the first time successfully applied in the IC measurement of metabolic CO_2 in a 3-D emulsion system containing bacterial cells with a hydrophobic substrate. Bioconversion of a hydrophobic substrate using bacterial cells has attracted much interest during the past decades because such technology is considered cleaner than conventional chemical processes (Schmid *et al*., 2001). However, most bacterial cell surfaces are hydrophilic and may limit their use as whole-cell biocatalysts for oil-like substrates. Enabling the industrially important and generally used bacteria, such as *E. coli*, *P. putida* and *L. Lactis*, to access water-insoluble substrates would extend their applications in both bioconversion and bioremediation. Our original research has demonstrated that a bacteria-chitosan network has an excellent ability to stabilize oil droplets in an aqueous solution, and allows the formation of a 3D scaffold of bacteria interfaced emulsion (Archakuna korn *et al*., 2015; Wongkongkatep *et al*., 2012). The bacteria interfaced emulsion has also been successfully developed into a biocatalyst for effective degradation of petroleum hydrocarbons (Gong, Li, Bao, Lv, & Wang, 2015) and for bacterial imprinting at the interface (Shen *et al*., 2014). To achieve efficient bioconversion, process control is inevitable and the biological status of the bacterial cells in the bacteria interfaced emulsion is important to quantify and sense. In this study, GC and IC techniques coupled with the standard culture technique were used to differentiate the real biological status of bacterial cells when they are in contact with toluene, *n*-hexane, *n*-decane, or soybean oil. Toluene is an industrially important aromatic hydrocarbon (C_7H_8) that is widely used as a cleaning solvent in the manufacturing of paints and coatings, adhesives, resins, and leather industry. The *n*-hexane (C_6H_{14}) has been used as an organic solvent in extraction of cooking oils, production of furniture, painting, and in rubber, pharmaceuticals, perfumes, footwear, leather and textiles. The *n*-decane ($C_{10}H_{22}$) was employed because of its intermediate length of carbon chain, while soybean oil is one of the most widely consumed cooking oils. Together with the standard culture technique, this commonly accessible instrumental analysis allowed us to distinguish the VBNC cells from culturable and metabolically active (live) cells, dead cells, and dormant cells. Therefore, GC and IC techniques are simple and useful tools that enable precise bioprocess control. These simple and rapid methods, using GC and IC techniques for the detection of VBNC state of the bacterial cells, can also be used to assure the biological safety of a manufacturing process in food or pharmaceutical industries.

2. Materials and Methods

2.1 Microorganisms and culture conditions

The bacterial strains used in this study included *E. coli* DH5α, *P. putida* F1 and *L. lactis* IO-1. *E. coli* DH5α and *P. putida* F1 were cultivated in LB (Lab M, Lancashire, UK) at 37°C and 30°C, respectively. *L. lactis* IO-1 was cultivated in MRS (Lab M, Lancashire, UK) at 30°C. An overnight culture was used to inoculate 200 ml of either LB or MRS (in 1 l-flask) to an initial optical density at 660 nm (OD_{660}) of 0.1. *E. coli* DH5α, *P. putida* F1 and *L. lactis* IO-1 were harvested at an early stationary phase with an OD_{660} of 2.4, 2.6 and 1.0, respectively by centrifugation at 2,760×g, 4°C for 15 min. Cell pellets were washed twice using 200 ml of sterile 0.1 M sodium phosphate buffer (pH 6.8) and re-suspended in the same buffer to the final OD_{660} of 35, 12 and 35 for *E. coli* DH5α, *P. putida* F1 and *L. lactis* IO-1 (corresponding to a cell concentration of 1 x 10^9 cfu/ml, respectively).

2.2 Preparation of the bacteria interfaced emulsions

White chitosan powder was available from Sigma-Aldrich with Product No. C3646 and Lot No. 068K00851 with deacetylation degree of 96% and MW of 1.6 x 10^6, and this was dissolved in 1% (v/v) acetic acid solution to prepare 1% (w/v) chitosan stock solution. The mixture was stirred continuously at room temperature for 24 h until a clear solution was obtained. The stock solution was then sterilized at 121ºC for 15 min and diluted as needed with sterile distilled water. Then, three volumes of bacterial cells suspended in 0.1 M sodium phosphate buffer solution (pH 6.8) were mixed with one volume of the 0.3% (w/v) chitosan solution. This aqueous phase was mixed with an oil phase (soybean oil, *n*-decane, *n*-hexane or toluene) at a volume ratio of 1:1. To obtain the bacteria interfaced emulsion, the mixture was homogenized using a homogenizer (IKA® T18 basic, ULTRA-TURRAX®) at 11,200 rpm for 3 cycles (30 sec/cycle with 2 min rest). The stability of the emulsion was evaluated as %Emulsification Index (%EI) or the percentage that emulsion phase volume represents in total volume of the mixture. Cell viability was determined by staining the bacteria interfaced emulsion with fluorescein diacetate (FDA) as reported by Capozzi *et al*. (2016). The emulsions were kept at room temperature and sampled at 0.5, 1, 2, 3, 4, and 5 days, then stained with FDA for 15 min, at a final concentration of 15 mM, in 500 mM sodium phosphate buffer (pH 7.0). The suspension of FDA-stained cells was imaged with an epifluorescence microscope (Olympus BX 50, Japan).

2.3 GC technique for monitoring metabolic CO_2

The emulsion, the positive control containing the same amount of bacterial cells as in the emulsion, and the negative control consisting of sterile 0.1 M sodium phosphate buffer (pH 7.4) (2 ml each) were transferred into 4 ml gas-tight vials. At a specific time, one vial was sacrificed and the

headspace (1 ml) was withdrawn and analyzed by GC (GC-2014, Shimadzu, Japan) equipped with ShinCarbon ST 80/100 (2 m in length, 2 mm inner diameter; 1/8 inch outer diameter) and a thermal conductivity detector. Helium was used as the carrier gas at a flow rate of 10 ml/min. The column temperature was raised from 120°C to 250°C at a rate of 20°C/min. The temperature for injector and TCD were set at 100°C and 280°C, respectively. The culturable cells were evaluated using a conventional plate count.

2.4 IC technique for monitoring metabolic CO_2

The prepared emulsion (2 ml) was transferred into a 4 ml-vial, which was placed inside a 25 ml-vial containing 10 ml of 0.007 M (equivalent to 0.4 g/l) standardized KOH. The accumulation of metabolic CO_2 was inferred from the conductivity of the KOH solution, measured using a commercial conductivity probe (CON-BTA, Vernier, USA; detection range 0-2000 µS/cm). The signal was recorded every 15 s with a resolution of 1 µS/cm. Untreated bacterial cell suspension was used as a positive control while the sterile buffer was used as a negative control. IC change was calculated as follow.

$$\text{IC change} = \kappa_i - \kappa_t$$

where κ_i is the initial conductance of the KOH solution and κ_t is the time-dependent conductance of the KOH solution after reacting with CO_2.

2.5 Statistical analysis

All experiments were performed in at least 2 replicates and statistical analysis was performed using Minitab software (Release 15, State College, PA).

3. Results and Discussion

3.1 Enumeration of culturable bacterial cells in 3-D scaffold of emulsion

The growth profiles of *E. coli* DH5α, *P. putida* F1 and *L. lactis* IO-1 were constructed and the cells were harvested at the early stationary phase. Oil-in-water emulsions with each of these 3 strains were obtained after blending with chitosan and emulsification with soybean oil, decane, hexane or toluene, as shown in Figure 1. Data on %EI were analyzed as an instance of Factorial Experiment. The results confirmed significant effects of 'Type of microorganism' (p-value = 0.044) and 'Type of solvent' (p-value = 0.000) but not of 'Day' (p-value = 0.920) on %EI. As there were significant interactions between 'Type of microorganism' and 'Day' (p-value = 0.023) and between 'Type of solvent' and 'Day' (p-value = 0.034), the data were analyzed further using the General Linear Model (GLM) option of a Minitab software. Interestingly, while the significant effect of 'Type of microorganism' on %EI could be observed on Day 1 (p = 0.027), the effect was insignificant on Day 5 (p = 0.307). On the other hand, the significant effect of 'Type of solvent' could be observed throughout the entire experiment. Overall, the emulsion prepared with soybean oil provided the highest %EI (85-94%), which is in a good agreement with our recent report (Hanpanich, Wongkongkatep, Pongtharangkul, & Wongkong-katep, 2017), whereas hexane showed the lowest %EI (69-78%), possibly due to its low boiling point compared with the other oils tested in this study (hexane 69°C, decane 174°C, toluene 111°C, smoke point of soybean oil 238°C). Consequently, large extent of hexane vaporization during homogenization could be expected, and may have caused the lowest %EI observed.

The partition coefficient (P) is defined as the ratio of concentrations of a target solvent between two immiscible liquids, generally water and *n*-octanol, and is abbreviated as $P_{o/w}$. The $P_{o/w}$ represents hydrophobicity of the target solvent. Solvents with log $P_{o/w}$ exceeding 4 are generally biocompatible, while those with lower log $P_{o/w}$ are not (Laane, Boeren, Vos, & Veeger, 1987). The solvents used for emulsion preparation in this study covered a broad range of log $P_{o/w}$ providing systems with different levels of metabolic activity and culturability, with toxicity predicted from log $P_{o/w}$ values from highest to least: toluene > hexane > decane > soybean oil. The results from the standard culture technique confirmed this ranking. The culturable cells of *E. coli* DH5α were retained at approximately 10^9 cfu/ml in the positive control, where the cells were suspended in the buffer without any treatment over the 5 days of study (Figure 3A), while a slight decrease in culturable cells was observed for *P. putida* F1 (Figure 3B).

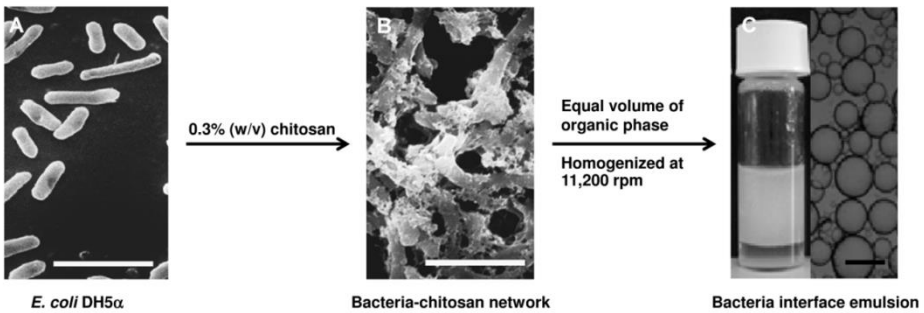

Figure 1. Bacteria interfaced emulsion preparation scheme. SEM images of *E. coli* DH5α before (A), and after network formation with chitosan (B). Scale bars (white) represent 3 µm in the SEM images. Appearance and micro-image of 3-D scaffold in bacteria interfaced emulsion (C). Scale bar (black) represents 100 µm.

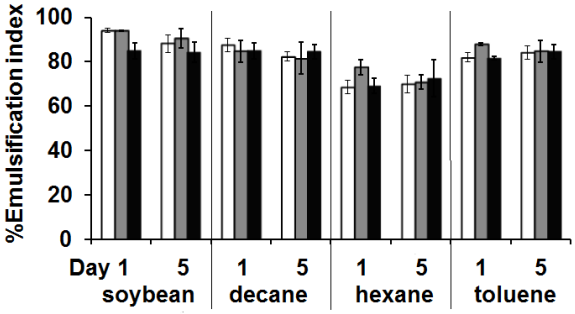

Figure 2. %Emulsification index (EI) of the emulsion prepared using *E. coli* DH5α (white), *P. putida* F1 (gray), and *L. lactis* IO-1 (black) stored at room temperature for 1 or 5 days.

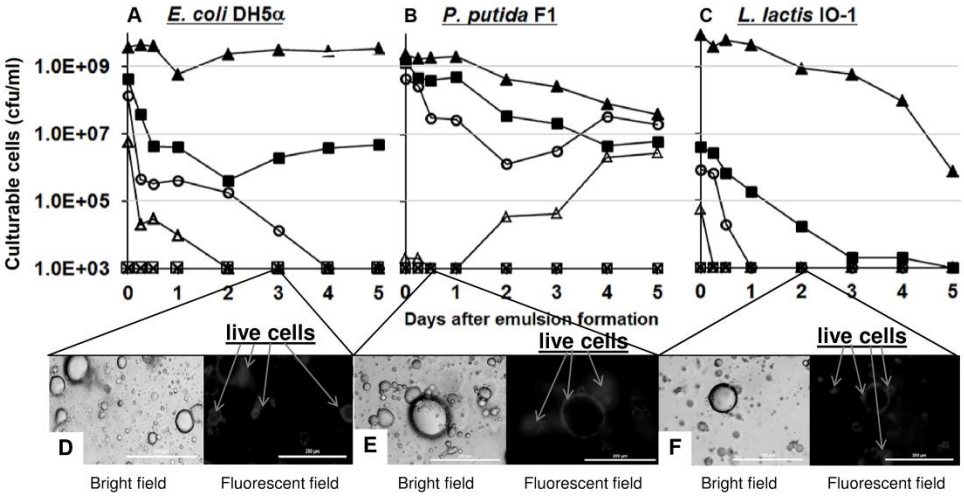

Figure 3. Culturable cells count of *E. coli* DH5α (A), *P. putida* F1 (B) and *L. lactis* IO-1 (C) in emulsion prepared with soybean oil (black squares), decane (white circles), hexane (white triangles) and toluene (white squares). The same amount of cells in the suspension without any oil phase is used as a positive control (black triangles) and the abiotic 0.1 M sodium phosphate buffer (pH 7.4) is used as a negative control (crosses). Fluorescent images of the emulsions provided by *E. coli* DH5α (D), *P. putida* F1 (E) and *L. lactis* IO-1 (F) using fluorescein diacetate staining. The fluorescence observed from the surface of emulsion indicates viable cells with active metabolism.

L. lactis IO-1, which is lactic acid bacteria generally used in fermented food that are claimed to have several health benefits (Chaikham, 2015; Kantachote, Ratanaburee, Hayisama-ae, Sukhoom, & Nunkaew, 2017; Phuapaiboon, Leenanon, & Levin, 2013), seemed to be the most sensitive strain in this study with over 3-log reduction in culturable cells observed in the positive control within 5 days (Figure 3C). After treatment with chitosan and homogenization with the oil phase, the numbers of culturable cells of *E. coli* DH5α and *P. putida* F1 gave the following order: soybean oil > decane > hexane > toluene, which matches the reversed order of toxicity of solvents predicted from log $P_{o/w}$ (Laane *et al.*, 1987). A similar trend was also observed in the case of *L. lactis* IO-1.

The viability of the bacteria located at the oil droplet interfaces was assessed by staining with fluorescein diacetate, which is non-fluorescent in metabolically inactive cells. If the cells are viable, indicated by active membrane esterase during metabolism, non-fluorescent fluorescein diacetate will be cleaved liberating the fluorescent fluorescein, so that bright fluorescence is observed in the fluorescence channel of the epifluorescence microscope. In this study, the bright fluorescence of fluorescein was observed from the surface of the emulsion, indicating viable cells at the interfaces, even when the culturable cell count was below the detection limit of 10^3 cfu/mL (Figure 3D-F).

3.2 Detection of VBNC state of bacteria in 3-D emulsion by GC and IC

GC has been considered a rapid determination method for the number of viable bacterial cells since 1987 (Guerzoni *et al.*, 1987), and the technique was developed for *in situ* determinations of bacterial growth, as reported in 2008 (Chai *et al.*, 2008). GC measures the CO_2 peak area corresponding to its concentration, while IC is the measurement of conductivity change in KOH solution upon CO_2 absorption. IC has several advantages over GC as it offers an opportunity for real-time monitoring with inexpensive equipment. A key design parameter affecting sensitivity of the IC method is concentration of the KOH solution. In this study, the average

conductivity of KOH solution was measured as a function of concentration and is presented in Table 1.

When bacterial cells are in contact with toxic chemicals, their metabolic activity can be suppressed, leading to low CO_2 production. Too high concentration of KOH will reduce the sensitivity in quantitative analysis of CO_2, especially when only a small amount of metabolic CO_2 is expected. On the other hand, a low concentration KOH solution may not be suitable as CO_2 is absorbed from ambient air, as expressed by the chemical reactions below (Taok *et al*., 2007), as this would reduce the conductivity beyond sensitivity of the equipment. In this study, the optimum concentration of 0.4 g/l KOH with average conductivity of 1,338 μS/cm was used, corresponding to the medium sensitivity of the commercial portable conductivity probe with a detection range of 0-2,000 μS/cm and a resolution of 1 μS/cm.

$$CO_2(g) + H_2O(l) \rightarrow H_2CO_3\ (aq)$$

$$H_2CO_3(aq) + 2OH^-(aq) \rightarrow CO_2^{3-}\ (aq) + 2H_2O(l)$$

In the positive control prepared without chitosan and organic phase, %metabolic CO_2 (GC) increased sharply during the first 12 h and then slowly increased until it reached 100% for all species of bacteria tested (Figure 4), where the 100% of metabolic CO_2 refers to the highest CO_2 production detected in the positive control over 5 days. In general, more culturable cells would give higher %metabolic CO_2 observed by GC and IC. However, there were very clear exceptions to this, especially in emulsion systems of *P. putida* F1 prepared with soybean oil or decane, because these had the highest metabolic CO_2 among the tested strains and even higher than that of the positive control (Figure 4B, E). It should be noted

Table 1. Average conductivities at various KOH concentrations

KOH concentration (g/l)	Average conductivity (μS/cm)
0.1	353
0.4	1,338
0.5	2,165
1	4,311
5	18,693

that the number of culturable cells in a solvent emulsion systems did not exceed that of the positive control (Figure 3). The results clearly indicate the presence of VBNC *P. putida* F1 cells in the 3-D scaffold of emulsion. Furthermore, this result indicates that *P. putida* F1 exhibited a high tolerance towards a wide range of organic solvents, as reported elsewhere (Lee, Park, Kim, Yoon, & Oh, 2002; Li *et al*., 2015), more than other bacteria including *E. coli* DH5α and *L. lactis* IO-1 tested in this study. Moreover, *P. putida* F1 seemed to be able to recover from hexane exposure as indicated by a continuous increase of metabolic CO_2 obtained by both GC and IC (Figure 4B, E) and the recovery of the culturable cells number from day 1 to day 5 (Figure 3B). This result agrees well with a previous report, in which *P. putida* F1 cells could recover from hexane shock due to an unknown innate ability or environmental factors (Heipieper & de Bont, 1994; Huertas, Duque, Marques, & Ramos, 1998). Panikov *et al*. (2015) reported that proteomic analysis showed upregulated (transporters, stress response, self-degrading enzymes and extracellular polymers) and downregulated (ribosomal, chemotactic and primary biosynthetic enzymes) proteins during VBNC state of *P. putida*.

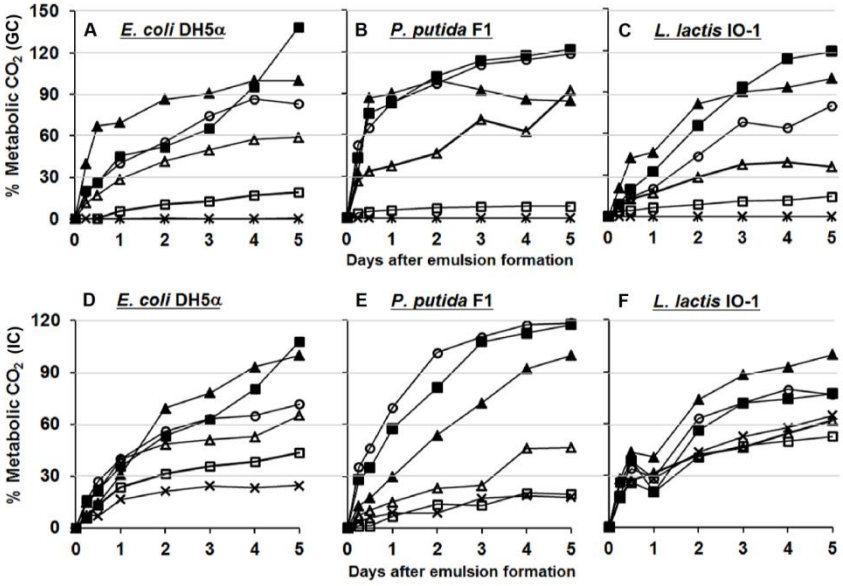

Figure 4. %Metabolic CO_2 of *E. coli* DH5α (A, D), *P. putida* F1 (B, E) and *L. lactis* IO-1 (C, F) evaluated by GC (upper panels) and by IC change (lower panels) in emulsions prepared with soybean oil (black squares), decane (white circles), hexane (white triangles) and toluene (white squares). The same amount of cells in the suspension without any oil phase is used as a positive control (black triangles) and the abiotic 0.1 M sodium phosphate buffer (pH 7.4) is used as a negative control (crosses).

L. lactis IO-1 was apparently the most sensitive of the strains tested, because the culturable cell count gradually decreased to a level below the detection limit at day 3, even in the least toxic soybean oil-emulsion (Figure 3C). The rank order of solvent toxicity toluene > hexane > decane > soybean oil was most obvious in the case of *L. lactis* IO-1, matching the highest to lowest metabolic CO_2 values recorded by GC when the cells were in contact with soybean oil, followed by decane, hexane and toluene (Figure 4C). Therefore, instrumental analysis of metabolic CO_2 by GC and IC coupled with conventional plate counts suggested the presence of VBNC state in *L. lactis* IO-1 cells in the emulsion system, which is in good agreement with the fluorescein diacetate staining results shown in Figure 3F. In fact, the VNBC state of lactic acid bacteria has been previously reported during the storage of wine (Millet & Lonvaud-Funel, 2000; Rizzotti *et al*., 2015).

4. Conclusions

The metabolic activity of *E. coli* DH5α, *P. putida* F1 and *L. lactis* IO-1 after exposure to toluene, hexane, decane or soybean oil in a 3-D scaffold of bacteria interfaced emulsion, was evaluated using conventional plate counting in combination with instrumental analyses of metabolic CO_2. These GC and IC techniques enabled the detection of VBNC state for all three strains tested, when exposed to hexane or decane. The ability of *P. putida* F1 to recover from stress shock during emulsification could be clearly observed from conventional plate counts, and from metabolic activity detected by GC or IC. Other methods for the detection of VBNC state of bacteria as summarized by Davis (2014), such as fluorescent in situ hybridization (FISH), real-time quantitative PCR (RT-qPCR or qPCR), reverse transcriptase (RT-PCR), propidium monoazide-PCR, and flow cytometry (FC)/ fluorescent activated cell sorting (FACS), all depend on expensive fluorescent dye/labeling techniques and on sophisticated instruments. The common GC and simple IC techniques for the detection of VBNC state of bacterial cells were demonstrated for the first time in this study, and can serve as standard and basic instrumental analyses to detect VBNC state of bacteria in various applications. The application of metabolically active *P. putida* and *E. coli* in bioconversion using the bacteria interfaced emulsion system is now being investigated.

Acknowledgements

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