Aromatic and aliphatic hydrocarbon consumption and transformation by the styrene degrading strain

*Pseudomonas putida* CA-3

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Abstract

*Pseudomonas putida* CA-3 is capable of consuming a number of aromatic and aliphatic hydrocarbons. With the exception of styrene none of the alkenes tested are capable of supporting the growth of *P. putida* CA-3 as sole sources of carbon and energy. The highest rate of alkene consumption was observed with styrene as the substrate. A 6.5- and 15.5-fold lower rate of substrate consumption was observed with indene and indole with the concomitant formation of 2-indanone and indigo, respectively. The presence of a sulphur (benzothiopene) or oxygen (benzofuran) in the cyclopentene ring resulted in further decreases in the rate of substrate consumption by whole cells of *P. putida* CA-3. *P. putida* CA-3 is incapable of consuming benzene and consumes toluene at a low rate. No detectable products were observed in supernatants of cultures incubated with benzothiopene, benzofuran or toluene. The aliphatic alkenes 1-octene and 1,7-octadiene were both consumed by whole cells of *P. putida* CA-3 at a rate equivalent to indene consumption. The consumption of (R) styrene oxide was 1.7- and 1.25-fold higher than that of the S isomer and the racemic mix, respectively. The rate of racemic indene oxide, 1,2-epoxyoctane and 1,2-epoxy-7-octene consumption was lower than their equivalent alkene and 55-, 11.8-, and 27.5-fold lower than the rate of racemic styrene oxide consumption. A transposon mutant incapable of growth with styrene or styrene oxide failed to transform indole to indigo. The ratio of styrene utilisation relative to other substrates changes in the mutant strain compared to the wild-type strain, e.g., Indene biotransformation by mutant AF5 is 1.9-fold higher than styrene consumption compared to the wild-type strain CA-3 where the rate of styrene consumption is 6.7-fold higher than indene consumption. This trend is also observed for other alkenes and epoxides.

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Keywords: Alkenes; Epoxides; Styrene monooxygenase; Styrene oxide isomerase; Whole cell biotransformation

1. Introduction

Pseudomonads have been identified as important agents for the biodegradation of aromatic compounds and modification of hydrocarbons to useful products

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The ability of microorganisms to transform these substrates is due in part to the presence of oxidoreductase enzymes, which transform the hydrophobic compounds into more hydrophilic and/or less reduced substrates [4]. The ability of microorganisms expressing oxidoreductases, such as styrene monooxygenase and naphthalene dioxygenase, to transform aromatic substrates, structurally related to the natural substrate, to value added products has been reported [1–5]. McCay et al. [7] have also reported on the transformation of aliphatic alkenes to epoxides by *Escherichia coli* expressing toluene-4-monoxygenase. However, very little is known about the ability of styrene degrading strains to consume or transform aromatic alkenes or their epoxides [1–3]. Furthermore, there are no reports on the transformation of aliphatic alkenes and epoxides by microorganisms expressing styrene monooxygenase.

*Pseudomonas putida* CA-3 possesses a styrene monooxygenase enzyme activity and is capable of growth with the aromatic alkenes styrene as a sole source of carbon and energy [8]. The pathway for styrene metabolism has been elucidated and involves the conversion of styrene via styrene epoxide, phenylacetaldehyde and phenylacetic acid [8]. The latter is converted to phenylacetyl-CoA by phenylacetyl-CoA ligase [9,10]. The biochemical pathway for styrene degradation after phenylacetyl-CoA in *P. putida* CA-3 has not been elucidated.

In this study, we examine the ability of *P. putida* CA-3 to consume aromatic and aliphatic alkenes as well as a number of their oxide derivatives and we identify any products that accumulate during consumption of these substrates. Furthermore, we examine the role of styrene degrading enzymes in the transformation of alkenes and epoxides by creating a transposon mutant incapable of growth with styrene or styrene epoxide and determining the rate of alkene and epoxide consumption by this mutant.

2. Materials and methods

2.1. Media, strains and plasmids

*Pseudomonas putida* CA-3 was cultivated in E2 medium [11] with one of these test substrates as a single source of carbon and energy. Cultures of *E. coli* for transposon mutagenesis were cultivated in LB media. *E. coli* CC118 *λpir* a donor strain for mutagenesis, contained the plasmid pUT mini-km1, which possesses the transposon Tn5 encoding kanamycin resistance [12]. Media for CC118 *λpir* pUT mini-km1 was supplemented with 50 μg kanamycin ml⁻¹. The helper strain *E. coli* HB101 contained a helper plasmid pKR600 which was required to deliver the plasmid from the donor strain to the recipient (*P. putida* CA-3) [12]. Media for *E. coli* HB101 (pKR600) was supplemented with chloroamphenicol (30 μg ml⁻¹). Both *E. coli* strains were kindly donated by Professor Victor de Lorenzo, Centro Nacional de Biотecnologia CSIC, Madrid, Spain.

2.2. Chemicals

All chemicals were of the highest quality commercially available and were purchased from Sigma–Aldrich. With the exceptions of 1-2, epoxy-7-octene, purchased from Lancaster synthesis (UK), and indene oxide purchased from Advanced Synthesis Technologies S.A. (USA). All chemicals were used without further purification.

2.3. Growth of strains

In the determination of the growth substrate range of *P. putida* CA-3 cells were cultivated overnight with styrene as the sole source of carbon and energy at 30 °C in a 1-l shake flask (200 r.p.m.) containing 200 ml E2 growth medium. These cells were then subcultured into fresh E2 medium with the test substrate. The hydrocarbon substrates in this study are volatile liquids that are poorly soluble in water. Therefore, the substrate (100–200 μl) was placed in a glass tube (10 mm in diameter × 60 mm in length) fused to the central base of the flask and transferred to the culture through the vapour phase. The optical density of the culture was then monitored over a 7-day period. Alkenes were also added directly to the liquid growth medium to theoretical concentrations of 5 mM and the change in optical density of the culture monitored over a period of 7 days.

For the maintenance of Tn5 mutants solid and liquid media were supplemented with kanamycin (50 μg ml⁻¹). *E. coli* strains were cultivated overnight at 37 °C in shake flasks (200 r.p.m.) containing LB and the required antibiotics. E2 and LB solid media were obtained by adding 1% (w/v) bacteriological agar. Initial overnight cultures of all the strains (5 ml), in their respective media, were used as master cultures for inoculation into larger flasks. Growth was determined by measuring optical density at 540 nm.

2.4. Whole cell biotransformation of alkenes

*Pseudomonas putida* CA-3 (wild-type) and strain AF5 (mutant) were grown overnight (14 h) on styrene and phenylacetic acid, respectively. Fourteen hours after inoculation fresh trace elements and styrene were added to a central tubing, fused to the base of the growth flask, and re-incubated at 30 °C for 2 h. Mutant cells (AF5) are not able to grow with styrene but the addition of styrene is performed in an attempt to induce enzymes capable of alkene and epoxide transformation. Cell suspensions were then washed with 50 mM potassium phosphate buffer, pH 7.0, and resuspended in the same
phosphate buffer to a final OD540 nm of between 2.0 and 5.0 depending on the substrate to be assayed. Two millilitres of cell suspension were added to a 125 ml screw top bottle containing 18 ml of 50 mM phosphate buffer, pH 7.0. Cells were incubated at 30 °C for 2 min before 2001 of alkene solution was added via an airtight mini-inert valve. All alkene stock solutions were dissolved in acetone to a final concentration of 100 mM. 1.5 ml liquid samples were removed from whole cell biotransformation using a syringe with a 15 cm needle that was inserted through the airtight mini-inert valve, allowing sampling without release of the alkene from the closed vessel. Samples were extracted with hexane/ethanol solution (ratio of 95:5) and the concentration of alkene determined by GC. All biotransformation controls showed less than 1% depletion of substrate concentration over a 30 min period. Indene was extracted into chloroform due to the low extraction efficiency of the potential product, indene oxide, into hexane/ethanol. The organic and aqueous phases were separated by centrifugation and the organic phase analysed by gas chromatography.

2.5. Gas chromatography

Alkenes, epoxides and their products were analysed using a Fison 8000 series GC equipped with an Agilent 19091Z-433/HP-1 capillary column, 30 m, 0.25 mm internal diameter, 0.25 μm film-coating (J&W Scientific, USA), hydrogen was the carrier gas and a temperature gradient of 50–140 °C was implemented with an increment of 10 °C min–1. Detection was by a flame ionisation detector.

2.6. Transposon mutagenesis

Conjugal transfer of the plasmid pUT mini-km1 from the donor strain to P. putida CA-3 was made by filter mating on LB agar. Overnight cultures of donor, recipient and helper cells were centrifuged at 6000 r.p.m. for 5 min and then washed twice with sterile 10 mM MgSO4 to eliminate any residual antibiotics. The pellets were resuspended in 0.5 ml of 10 mM MgSO4, 50 μl of each were mixed in equal ratio and placed on to a nitrocellulose membrane (0.45 μm pore size, Whatman®) on an LB agar plate. These conjugation plates were incubated overnight at 30 °C. The nitrocellulose membrane was transferred to 5 ml of 10 mM MgSO4 and the cell mix suspended by gentle mixing. The conjugation mix was spread on to E2 agar containing 5 mM phenylacetic acid, 50 μg kanamycin ml–1, and 1 mM indole. Selection plates were incubated and monitored over a period of five to six days. Resulting colonies were picked off and organised into 96 well microtitre plates[13]. Using a replicator these cultures were transferred on to E2 agar supplemented with kanamycin (50 μg ml–1) and indole (1 mM). Plates were then placed into desiccators containing styrene, styrene oxide, or phenylacetylaldehyde, respectively, to test the growth of the mutant strains.

3. Results and discussion

3.1. Growth of P. putida CA-3 with various alkenes

Pseudomonas putida CA-3 grows well with styrene as a sole source of carbon and energy [8]. In incubations with 250 μl of styrene supplied to 50 ml of E2 growth medium an optical density (540 nm) of 0.8 was achieved within 16 h of incubation. However, P. putida CA-3 failed to grow above an optical density at 540 nm of 0.03 after 7 days of incubation when any other alkene tested in this study were supplied as the sole source of carbon and energy.

3.2. Biotransformation of alkenes by P. putida CA-3

3.2.1. Aromatic alkenes

GC analysis revealed that P. putida CA-3 is capable of consuming a range of aromatic alkenes (Fig. 1). All bio-

![Fig. 1. The chemical structure of the aromatic and aliphatic substrates for P. putida CA-3. Styrene, indene, indole, benzothiopene, benzofuran, benzene, toluene, styrene oxide, indene oxide, 1-octene, 1,7-octadiene, 1,2-epoxyoctane, 1,2-epoxy-7-octene.](image-url)
transformation controls showed less than 1% reduction in substrate concentration in the absence of *P. putida* CA-3 cells. The highest rate of alkene consumption was observed when washed cells were incubated with styrene as the substrate (Table 1). The substitution of the vinyl side chain of styrene with a cyclopentene ring (indene) resulted in a 6.7-fold lower rate of substrate consumption (Table 1). While the product of styrene consumption, styrene oxide, did not appear due to the presence of other enzymes in *P. putida* CA-3, known to metabolise styrene to central metabolites [8–10], analysis of the biotransformation medium for indene revealed the accumulation of a single product 2-indanone from indene (Fig. 2(a)). All of the indene transformed appeared as 2-indanone (Fig. 2(a)). Previous studies have shown that *P. putida* S12, expressing styrene monooxygenase accumulates 2-indanone from indene [1]. However, neither the rate of reaction nor the stoichiometry of this biotransformation was reported. The transformation of indene by microorganism expressing dioxygenases has also been reported in the literature. A number of products including indene oxide, cis-(1R,2S)-indandiol, cis-(1S,2R)-indandiol, trans-indandiol, and 1-indenone have been reported [14–19].

Substitution of the carbon of indene with the nitrogen of indole resulted in a 2.3-fold decrease in the rate of substrate consumption (Table 1). The formation of indigo from indole by *P. putida* CA-3 has previously been described [1]. The substitution of carbon two of the cyclopentene ring with sulphur (benzothiopene, Fig. 1) resulted in a further decrease in the rate of substrate consumption (Table 2). Benzothiopene is poorly soluble in aqueous solution and was added to biotransformation media at lower concentrations than other aromatic alkenes tested in this study (Fig. 2(a)). Consequently, the consumption of benzothiopene was almost complete within 24 min (Fig. 2(a)). The presence of an oxygen moiety in the cyclopentene ring of benzofuran decreased the rate of substrate consumption by whole cells of *P. putida* CA-3 to almost non-detectable levels (Table 2, Fig. 2(a)). Previous studies have not reported on the activity of styrene monooxygenase towards substrates such as benzothiopene or benzofuran. However, the aerobic metabolism of benzothiopene has been reported for an enrichment culture with a *Pseudomonas* strains as the predominant microorganism [20]. From the data presented here there is an obvious effect of substitution on the rate of substrate depletion by *P. putida* CA-3 (Table 2). It is possible that the electronic and steric effects of these substitutions may be contributing to the rate of substrate depletion. However, it is also possible that substrate solubility and substrate uptake by the cell affect the rate of substrate consumption. In the future purification of the styrene monooxygenase enzyme should help to clarify these issues.

*Pseudomonas putida* CA-3 is incapable of growth with toluene or benzene. While benzene was not consumed, a low rate of toluene consumption was observed with washed cell suspensions of *P. putida* CA-3 (Table 1).

**Table 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (Units g cdw⁻¹)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Styrene</td>
<td>342 ± 36</td>
</tr>
<tr>
<td>Indene</td>
<td>51 ± 9</td>
</tr>
<tr>
<td>Indole</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>Benzothiopene</td>
<td>15 ± 1.5</td>
</tr>
<tr>
<td>Benzofuran</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Toluene</td>
<td>22 ± 6</td>
</tr>
<tr>
<td>Benzene</td>
<td>ND²</td>
</tr>
<tr>
<td>1-Octene</td>
<td>58 ± 2</td>
</tr>
<tr>
<td>1,7-Octadiene</td>
<td>47 ± 13</td>
</tr>
</tbody>
</table>

Unit = μmol per minute.
All data are the average of at least three independent determinations.
Less than 1% consumption of substrate was observed in abiotic control assays containing no cells over a 30-min period.

³ cdw. Cell dry weight.

⁴ ND, no detectable consumption of substrate.

**Table 2**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (Units g cdw⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Styrene oxide</td>
<td>330 ± 43</td>
</tr>
<tr>
<td>S-styrene oxide</td>
<td>139 ± 14</td>
</tr>
<tr>
<td>R-styrene oxide</td>
<td>421 ± 47</td>
</tr>
<tr>
<td>Indene oxide</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>1,2-Epoxyoctane</td>
<td>28 ± 12</td>
</tr>
<tr>
<td>1,2-Epoxy-7-octene</td>
<td>18 ± 3</td>
</tr>
</tbody>
</table>

Unit = μmol per minute.
All data are the average of at least three independent determinations.
No significant consumption of oxide substrates was observed in control assays containing no cells over a 30-min period.
Fig. 2(b)). Toluene does not have a vinyl component to its side chain and as such it is a significantly different substrate for whole cells of *P. putida* CA-3 compared to other substrates used in this study. The oxidation of toluene by cells expressing styrene monoxygenase could occur on the aromatic ring or the methyl side chain, potentially generating either an arene oxide or benzylalcohol, respectively. The arene oxide is highly unstable in aqueous solution and thus would form a phenol. However, no oxides, phenol products or benzylalcohol were detected by GC or HPLC analysis of biotransformation supernatants. This may be due to through conversion of the initial products to compounds that accumulate inside the cell or the formation of products that accumulated at levels below the detection limit of the analytical equipment.

The aliphatic alkenes 1-octene and 1,7-octadiene were both consumed by whole cells of *P. putida* CA-3 at a rate equivalent to indene consumption (Table 1, Fig. 2(b)). A slight lag period was observed for 1-octene but not 1,7-octadiene consumption (Fig. 2(b)). However, the rate of 1-octene consumption is 1.2-fold higher than 1,7-octadiene (Table 2). A transitory product, identified as 1,2-epoxyoctane was sometimes identified in the biotransformation of 1-octene (data not shown). However, this product did not reproducibly appear in biotransformations and thus is not represented in Fig. 2. No products were detected in biotransformations of 1,7-octadiene (data no shown). The formation of epoxides from aliphatic alkenes is not widely reported [7,21]. However, 7,8-epoxy-1-octene and 1,2-7,8-epoxyoctane formation from 1,7-octadiene has been described for a single strain of *Pseudomonas oleovorans* [21]. The lack of products from whole cell biotransformation assays suggests the through conversion of the likely intermediates (epoxides) to products that are not detectable by HPLC or GC analysis.

### 3.2.2. Aromatic and aliphatic oxide consumption by whole cells of *P. putida* CA-3

While many studies have focused on the biological formation of epoxides from arenes fewer studies have looked at epoxide consumption by bacteria [22–26]. While *P. putida* CA-3 is capable of consuming styrene epoxide and indene oxide there is a dramatic (55-fold) difference in the rate of consumption (Table 2). This may suggest that indene epoxide is toxic to the cell. A lag period was observed before consumption of indene oxide by washed cell suspensions of *P. Putida* CA-3 (Fig. 3). The difference in the rate of indene oxide and indene consumption (Tables 1 and 2) would suggest that indene oxide could accumulate from indene. The appearance of 2-indanone (Fig. 2) and not indene oxide in the biotransformation of indene is possibly due to the spontaneous chemical conversion of indene oxide to 2-indanone. However, indene oxide did not appear to be unstable in abiotic aqueous controls as 2-indanone formation was not detected. The formation of indene oxide inside in bacterial cells may affect the stability of indene oxide or make is prone to attack by other enzymes inside in the cell.

Washed whole cell suspensions of *P. putida* CA-3 consumed racemic styrene oxide (Table 2) at a rate equivalent to styrene (Table 1). Due to the commercial availability of single enantiomers of styrene oxide the consumption of these substrates by whole cells of *P. putida* CA-3 was tested. The consumption of the *R* isomer of styrene oxide was just over 3 and 1.25-fold higher than that of the *S* isomer and the racemic mix, respectively (Table 2). The lower rate of substrate consumption for the racemic mix compared to the rate of (R)-styrene oxide consumption would suggest that the *S* and *R* isomers may be competing for the same enzyme(s) acting on these substrates. While the microbial metabolism of epoxides has been examined to our knowledge a comparison of the rate of racemic styrene epoxide, *R*-styrene oxide and *S*-styrene epoxide by styrene degrading bacteria has not previously been determined [22,24–26].

In addition to aromatic substrates *P. putida* CA-3 is also capable of consuming the aliphatic oxides 1,2-epoxyoctane and 1,2-epoxy-7-octene albeit at rates 11.8- and 18.3-fold lower than racemic styrene oxide (Table 2, Fig. 3). The lower rates may reflect the substrate specificity of the enzyme transforming the epoxides. Interestingly the consumption of 1,2-epoxyoctane and 1,2-epoxy-7-octene ceased after 18 and 12 min, respectively. This inhibition may be due to accumulation of the predicted aldehyde products. However, there were no products observed by HPLC or GC analysis and thus the inhibition of enzyme activity by epoxides may be a more likely explanation. While styrene grown cells of *P. putida* CA-3 consume a range of alkenes and oxides it is not clear if the enzymes responsible for styrene metabolism are involved in their consumption. To investigate the role of
sterene degrading enzymes in the consumption of alkenes and oxides used in this study a transposon mutant of \textit{P. putida} CA-3 was generated.

3.2.3. Generation of mutants of \textit{P. putida} CA-3 incapable of growth with styrene

\textit{Pseudomonas putida} CA-3 transposon mutants were tested for (A) growth on styrene, styrene oxide, phenylacetaldehyde and phenylactic acid and (B) the ability to transform indole to indigo. Mutant strain AF5 was incapable of growth with styrene or styrene oxide but grew normally with phenylacetaldehyde or phenylactic acid as the sole source of carbon and energy. Furthermore, the mutant and wild-type strain when grown under the same conditions (5 mM phenylactic acid) and then incubated with phenylactic acid or phenylacetaldehyde consume oxygen at the same rate (data not shown). Strain AF5 failed to transform indole to indigo (after one week of incubation on an agar plate). It has been shown in previous studies that indole biotransformation by \textit{P. putida} CA-3 is dependent on both styrene monooxygenase and styrene oxide isomerase \cite{1}. The rate of styrene and styrene oxide transformation by the mutant was 38- and 15-fold lower than wild-type CA-3 (Table 3). Given that this mutant can not accumulate indigo from indole and it is incapable of growth with styrene or styrene oxide epoxide it is surprising that it was still capable of consuming styrene and styrene epoxide (albeit at very low rates) as well as other alkenes and epoxides. These results may suggest that an incomplete knockout or a mutation in a regulatory gene was generated allowing for residual styrene monooxygenase and styrene oxide isomerase activity to remain in the mutant. However, the other possibility is that another enzyme system is capable of transforming alkenes and epoxides. Alterations in the rate of styrene utilisation relative to other alkenes in the mutant strain compared to the wild-type strain may support this postulation. The rate of indene biotransformation by mutant AF5 is 1.9-fold higher than styrene consumption. This is dramatically different from the wild-type strain where the rate of styrene consumption is 6.7-fold higher than indene consumption. This trend is again observed for octene, 1,7-octadiene where the rate of substrate consumption by the mutant strain is higher than styrene consumption (Table 3). Furthermore, while the rate of styrene consumption by the wild-type strain is 38 and 36-fold higher than the rate of indene epoxide and toluene consumption, respectively, the rate of indene epoxide, toluene, and styrene consumption is almost identical in the mutant strain. These results suggest that another enzyme system may play a role in their degradation by \textit{P. putida} CA-3 as a leaky mutation or a regulatory knockout should not affect the relative rates of substrate consumption by mutant cells. If another enzyme system is operating then it appears that this is partially responsible for aromatic alkene consumption and responsible for nearly half the rate of aliphatic alkene and epoxide consumption by \textit{P. putida} CA-3.

While \textit{P. putida} CA-3 accumulates products from some substrates the lack of products from other substrates has potentially detrimental effects on the development of \textit{P. putida} CA-3 as a biocatalyst for the transformation of alkenes to epoxides due to the potential for mixed product formation and product through conversion.

Acknowledgement

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\cite{5} Allen, C.C.R., Boyd, D.R., Larkin, M.J., Reid, K.A., Sharma, N.D. and Wilson, K. (1997) Metabolism of naphthalene, 1-

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<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (Units g cdw−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Styrene</td>
<td>9 ± 6</td>
</tr>
<tr>
<td>Styrene epoxide</td>
<td>22 ± 7.1</td>
</tr>
<tr>
<td>Indene</td>
<td>17.1 ± 5.6</td>
</tr>
<tr>
<td>Indene oxide</td>
<td>9.9 ± 2.5</td>
</tr>
<tr>
<td>Toluene</td>
<td>9.5 ± 3.4</td>
</tr>
<tr>
<td>Benzene</td>
<td>ND*</td>
</tr>
<tr>
<td>1-Octene</td>
<td>26.6 ± 5.6</td>
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<tr>
<td>Epoxyoctane</td>
<td>11.6 ± 2.7</td>
</tr>
<tr>
<td>1,7-Octadiene</td>
<td>20.3 ± 12.4</td>
</tr>
<tr>
<td>1,2-Epoxy-7-octene</td>
<td>8.2 ± 3.6</td>
</tr>
</tbody>
</table>

Unit = μmol per minute.

All data are the average of at least three independent determinations.

Less than 1% consumption of substrate was observed in control assays containing no cells over a 30-min period.

No significant consumption of oxide substrates was observed in abiotic control assays containing no cells over a 30-min period.

* ND, no detectable consumption.


