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Degradation of cyclohexane and cyclohexanone by *Bacillus lentus* strain LP32

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A Gram-positive bacterium, *Bacillus lentus* LP32, originally isolated on the basis of its ability to utilise pyrene as sole source of carbon was found to be able to grow luxuriantly on alicyclic compounds as sole substrates. It showed poor growth on anthracene, naphthalene, 1-naphthol and phenanthrene. Growth rate on cyclohexane was 1.32 d^{-1}, while doubling time was 0.76 d. The corresponding values for growth on cyclohexanone were 0.77 d^{-1} and 1.29 d, respectively. Within 10 days, the amount of cyclohexane in culture reduced from 317.62 to 102.55 mgl^{-1}, then to 23.04 mgl^{-1} on day 18. On cyclohexanone, substrate concentration decreased from 287.56 mgl^{-1} to 101.66 mgl^{-1} in 10 days before declining to 24.21 mgl^{-1} on day 18. The rate of degradation when growing on cyclohexane was 23.50 $mgl^{-1}d^{-1}$ in the first 10 days and 9.93 $mgl^{-1}d^{-1}$ between day 10 and day 18, with 67.71% degradation in 10 days and overall percentage degradation of 92.43%. On cyclohexanone, the corresponding values were 18.59 and 9.68 mg $l^{-1}d^{-1}$ as well as 64.65 and 91.58%, respectively. This organism is a potential candidate for bioremediation purpose.

Keywords: Degradation, cyclohexane, cyclohexanone, alicyclic compounds.

INTRODUCTION

Alicyclic compounds are major components of crude oil (Okoh, 2006). Their relative amount in the crude oil (20 to 70%) is dependent upon the nature and origin of the petroleum (Ilori, 1999; Maier, 2009). They usually find their way into soil and natural bodies of water, including aquifers, either accidentally or deliberately, during commercial operations such as oil drilling and oil transportation; or during industrial applications as solvents, though on a smaller scale (Ilori, 1999; Lee and Cho, 2008). They occur in nature as components of plant oils, paraffins, microbial lipids and pesticides (Maier, 2009).

Alicyclic compounds have been reported to be relatively persistent in the environment when compared to other components of the crude oil (Okoh, 2006). Indeed, they are reputed to be the saturated hydrocarbons most resistant to microbial attack (Sikkema et al., 1995; Ko and Lebault, 1999). Thus, their biological fate in the environment has been a major source of concern to environmentalists (Rio-Hernandez et al., 2003).

Reports on the use of axenic cultures of microorganisms to degrade alicyclic compounds are fewer in the literature compared with other fractions of petroleum (Mechichi et al., 2003; Amund et al., 2006). Their degradation is thought to occur primarily by commensalistic and cometabolic reactions under aerobic condition (Maier, 2009). The potential membrane toxicity of alicyclic compounds to microbial cells in little amounts and their low water solubility greatly account for the persistence in the environment (Sikkema et al., 1995; Amund et al., 2006).

It is equally noteworthy that most studies on the biodegradation of alicyclic compounds are based on

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Abbreviations: API, Analytical profile index; **MSM,** mineral salts medium; **TVC,** total viable count; **GC,** gas chromatography.

cyclohexane (Tonge and Higgins, 1974; Donoghue et al., 1976; Stirling and Watkinson, 1977; Anderson et al., 1980; Trower et al., 1985; Brzostowicz et al., 2000). Axenic cultures including *Nocardia* sp., *Pseudomonas* sp., *Xanthobacter* sp. have been reported to grow on cyclohexane (Stirling and Watkinson, 1977; Anderson et al., 1980; Trower et al., 1985). Similarly, a microbial consortium of *Stenotrophomonas* sp. and *Rhodococcus* sp. was reported to be able to utilise this substrate (Lee and Cho, 2008). The *Rhodococcus* sp. was further shown to be able to utilise cyclohexane as sole source of carbon and energy, producing as part of the intermediates cyclohexanone and cyclohexanol, 2-cyclohexene-1-one and phenol (Yi et al., 2011).

On the other hand, reports on degradation of cyclohexanone have been scantier than envisaged. Brzostowicz et al. (2000) reported the ability of *Brevibacterium* sp. strain HCU to utilise cyclohexanone as sole source of carbon and energy. According to the authors, the monooxygenases required to degrade cyclohexanone are only induced in the presence of the hydrocarbon. Amund et al. (2006) reported the ability of strains of *Pseudomonas*, *Acinetobacter*, *Arthrobacter* and *Norcardia* to grow on cyclohexanone and cyclohexanol. This degradative ability was found to be chromosomally borne. In this paper, we report the degradation of alicyclic compounds, cyclohexane and cyclohexanone, by a *Bacillus* species originally isolated on the basis of its ability to grow with pyrene as sole source of carbon and energy.

MATERIALS AND METHODS

Microorganism and culture condition

The organism used in this study was *Bacillus lentus* strain LP32. The isolation and characterisation of the organism by continuous enrichment of run-off soil adjacent to an asphalt plant in Lagos, Nigeria on the basis of its ability to use pyrene as sole source of carbon and energy has been documented elsewhere (Obayori, 2008). The isolate was maintained in glycerol:nutrient broth (1:1) at -20°C. It was checked for purity by plating onto nutrient agar and observing the 18 h old culture under the microscope. The identity of the isolate was reaffirmed by analytical profile index (API) using the API ZONE and API V.20 test kit according to the manufacturer's specifications (Biomerieux Inc., Durham, NC, USA).

Colonies growing on Luria-Bertani agar with very low percentage of pyrene (0.005%) were harvested with sterile inoculating loop, pooled and transferred to screw-capped bottles containing 5 ml of physiological saline (0.9% NaCl). Enough culture was transferred to achieve an OD_{520} of approximately 1.5. The organism was grown in mineral salts medium (MSM) previously described by Kastner et al. (1994). The medium contained per litre Na_2HPO_4, 2.13 g; KH_2PO_4, 1.30 g; NH_4Cl, 0.50 g and $MgSO_4.7H_2O$, 0.20 g. The pH of the medium was adjusted to 7.2. Trace elements solution (1 ml/l) described by Bauchop and Elsden (1960) was sterilized separately and added aseptically to the medium. Unless otherwise stated, incubation was performed at room temperature (27 ± 2.0°C). The stock culture was aerobically grown routinely in MSM containing cyclohexane (SIGMA-ALDRICH Inc., USA) and cyclohexanone (SIGMA-ALDRICH Inc., USA) as the sole carbon sources in separate conical flasks.

Substrate specificity of isolate

The isolate was tested for its ability to grow on the following substrates: anthracene, 1-naphthol, pyrene, cinnamic acid, succinic acid, biphenyl and naphthalene. Substrate specificity was evaluated in MSM broth supplemented with 50 mg L^{-1} of respective solid hydrocarbons or 0.1% (*V*/*V*) in case of liquid substrates as a sole carbon and energy source. Degradation was monitored by cell increase with reference to uninoculated flasks, coupled with disappearance of the crystals in the case of pyrene and anthracene. Inocula were 24 h LB-grown cultures and inoculation was carried out to achieve initial optical density of 0.05 (OD_{520}). Incubation was performed at room temperature (27 ± 2.0°C).

Time course of growth of isolate on cyclohexane and cyclohexanone

Replicate flasks containing 50 ml of autoclaved MSM with 0.5 ml of filter-sterilised cyclohexane or cyclohexanone were prepared. Thus for each substrate, there were 10 triplicate sets, making a total of 30 flasks per substrate. The flasks were inoculated to achieve an initial concentration of the total viable count (TVC) about 2.0 x 10^7 cfu ml^{-1} and incubated at room temperature (27 ± 2.0°C) for a period of 18 days.

Flasks inoculated with heat-inactivated cells served as controls. Total viable counts were determined at 2 days interval by plating out appropriate dilutions of the cultures onto nutrient agar. Mean generation times and specific growth rates were calculated using nonlinear regression of growth curves for the period when growth rates were maximal (Prism version 5.0, Graphpad software, San Diego, CA, USA).

Chromatographic determination of residual alicyclic substrates

Residual concentration of the alicyclic compound in the culture medium was analysed by gas chromatography (GC) (Hewlett-Packard) fitted with flame ionization detector (FID). An aliquot (20 ml) of the sample was extracted with 5 ml hexane and concentrated to 1 ml. One microlitre of the concentrated sample was injected into the chromatograph column. The column was OV-101 and SE 30 with length of 60 m. The injector and detector temperatures were maintained at 200 and 300°C, respectively. The initial temperature of 70°C and a final temperature of 320°C were used for the analysis which was programmed to rise at 10°C per min. Nitrogen was used as the carrier with the gas flow rate of hydrogen at 45 ml per min, nitrogen at 22 ml per min and air flow rate of 450 ml per min. The GC runs were carried out on the sample at day 0, day 10 and day 18.

RESULTS AND DISCUSSION

Identification and characterisation of isolate

LP32 was a Gram-positive, catalase and oxidase positive, spore forming, motile rod, with β-haemolysis on blood agar. It was positive for starch hydrolysis, liquefied gelatine and reduced nitrate. It utilised citrate, lactose, sucrose, glucose and fructose. It was negative for urease, indole and hydrogen sulphide production, and equally failed to grow on sorbitol, ducitol, mannitol,

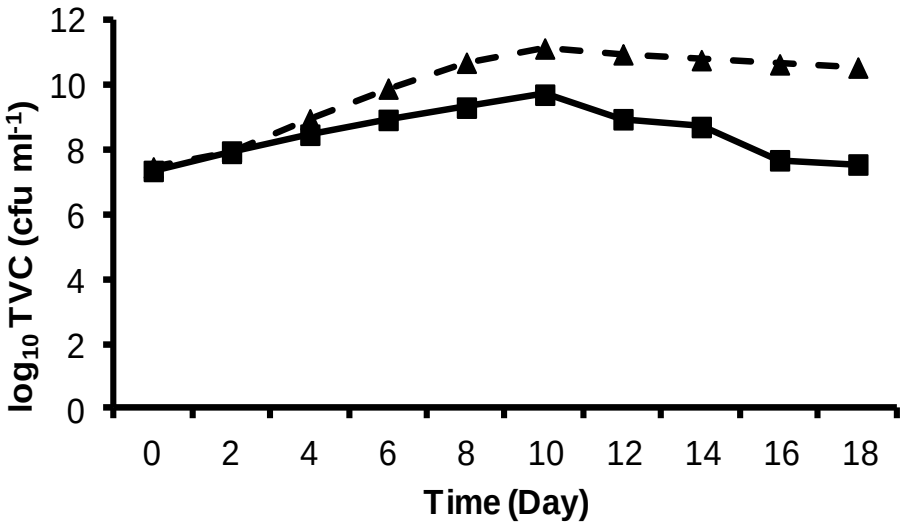

Figure 1. Time course of growth of *Bacillus lentus* strain LP32 on cyclohexane (▲) and cyclohexanone (■) in mineral salt medium (MSM) supplied with 0.1% percent of the respective substrates as sole source of carbon and energy. Incubation was carried out at room temperature (27 ± 2°C) with shaking at 150 rpm. Time point data are means of triplicate readings, error bars were eliminated for the purpose of clearity.

Table 1. Kinetics of degradation of alicyclic substrates by *Bacillus lentus* LP32.

Substrate	*K* (day $^{-1}$)	T (day)	DR_{10} (mg l^{-1} d^{-1})	DR_{18} (mg l^{-1} d^{-1})	PD_{10} (%)	PD_{18} (%)
Cyclohexane	1.32	0.76	23.50	9.93	67.71	92.43
Cyclohexanone	0.77	1.29	18.59	9.68	64.65	91.58

K, Growth rate; T, doubling time; DR_{10}, degradation rate in the first 10 days; DR_{18}, degradation rate between day 10 and 18; PD_{10}, percentage degraded in the first 10 days; PD_{18}, overall percentage degraded in 18 days. The substrates were supplied at a concentration of 0.1% (*V*/*V*) and separated on OV-101 column with length of 60 m.

arabinose and maltose. It was therefore putatively identified as *Bacillus lentus*. Substrate specificity showed that LP32 did not grow on biphenyl, and grew poorly on anthracene, naphthalene, 1-naphthol and phenanthrene. However, it showed heavy growth on cyclohexane and cyclohexanone.

Biodegradation of cyclohexane and cyclohexanone

The time courses of growth of *B. lentus* strain LP32 on cyclohexane and cyclohexanone are shown in Figure 1. On cyclohexane from an initial population density of 2.94 x 10^7 cfu ml^{-1} the organism concentration steadily increased to 3.27 x 10^{11} cfu ml^{-1} in ten days before dropping to 3.62 x 10^{10} cfu ml^{-1} on day 18. On the other hand, when grown on cyclohexanone population density increased from 2.3 x 10^7 to 5.28 x 10^9 cfu ml^{-1} on day 10 before declining to 3.59 x 10^7 cfu ml^{-1} on day 18. Growth rate on cyclohexane was 1.32 d^{-1} while doubling time was 0.76 d (Table 1) the corresponding values for growth on cyclohexanone were 0.77 d^{-1} and 1.29 d, respectively.

Kinetics of growth and degradation of alicyclic compounds (Table 1) showed that the rate of degradation when growing on cyclohexane was 23.50 $mgl^{-1}d^{-1}$ in the first ten days and 9.93 $mgl^{-1}d^{-1}$ between day 10 and 18, with 67.71% degradation in 10 days overall percentage degradation of 92.43%. When LP32 was grown on cyclohexanone the corresponding values were 18.59 and 9.68 mg $l^{-1}d^{-1}$ as well as 64.65 and 91.58%, respectively. The ability of *B. lentus* LP32 to degrade cyclohexane and cyclohexanone is of interest because *Bacillus* species had rarely been previously reported to play a major role in degradation of alicyclic compound. The only report on cyclohexane degradation by *Bacillus* till date was that of Lee et al. (2013). The authors isolated from loess four strains, *Bacillus cereus* strain VOC18, *Bacillus thuringensis* strain VOC 11, *B. thuringensis* strain VOC13 and *Bacillus megaterium* strain VOC 03 capable of utilizing or tolerating cyclohexane to varying extents.

However, strains of *Bacillus* have been previously and consistently reported as a hydrocarbon utilizers (Okerentugba and Ezeronye, 2003; Okoh, 2006; Nwaogu et al., 2008). Other organisms previously isolated from alicyclic compounds span genera such as *Pseudomonas, Norcardia, Brevibacterium, Acinetobacter, Xanthobacter, Alicycliphilus denitrificans and Arthrobacter* (Stirling and Watkinson, 1977; Anderson et al., 1980; Trower et al., 1985; Ilori, 1999; Brzostowicz et al., 2000; Mechichi et al., 2003; Amund et al., 2006).

The fact that LP32 which was originally isolated on pyrene was able to use alicyclic compounds and a variety of substrates as sole source of carbon is of interest. Partly because of differences in metabolic pathways, it is rare to find organisms which could degrade both aliphatic and aromatic compounds effectively. However, it has been suggested that long periods of exposure to mixture of hydrocarbon and preponderance of enabling intrinsic and extrinsic factors could lead to acquisition of such rare ability (Obayori et al., 2009). It is equally noteworthy that a degrader of alicyclics had been previously demonstrated to grow on pyrene, benzene and other diverse hydrocarbons and petroleum cuts (Lee and Cho, 2008). The steady increase in the population of organisms in both substrates between day 0 and 10 could be attributed to the utilisation of the sole carbon sources, cyclohexane and cyclohexanone (Okerentugba and Ezeronye, 2003; Amund et al., 2006). The observation of a downward trend in the total viable count of the organisms after day 10 could be attributed to nutrient exhaustion (especially the carbon source) or accumulation of toxic metabolites (Atlas, 1994). The main routes of degradation of cyclohexane and cyclohexanone metabolism involves the formation of 1-oxa-2-cycloheptane (*ε*-caprolactone) and 6-hydroxyhexanoate (6-hydroxycaproate) as intermediate products (Wagner et al*.,* 2002). These intermediate products are toxic to microorganisms that lack *ε*-caprolactonase and 6-hydroxyhexanoate dehydrogenase (Brzostowicz et al*.,* 2000). Thus, this may account for the downward trend of total viable count during the succeeding days.

The gas chromatography results further accentuated the result obtained from total viable counts. Over 67.71% of cyclohexane was metabolized by *B. lentus* within 10 days. This indicated that trend of degrading alicyclic compounds within the first ten days was better pronounced than the succeeding 11 days. In the same vein, 64.65% of cyclohexanone was degraded by *B. lentus* within the first ten days. The overall percentage degradation of cyclohexane of 92.2% is similar to the result obtained by Lee and Cho (2008) for *Rhodococcus* strain ECI which metabolised 9% hexane. The ability of strain LP32 to effectively degrade cyclohexane and cyclohexanone *in vitro* and its broad substrate susceptibility suggests that it could further investigation for application in production of value added bio-products from alicyclic compound and bioremediation of hydrocarbon polluted compartments.

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