SUMMARY

Biochemical and Geneticv Analysis of Gaseous Alkane Utilizing Bacteria

Biochemical and genetic aspects of methane and higher chain alkanes (carbon chain length five or more), both aliphatic as well as aromatic, have been quite extensively investigated, but very less was known about pathways and genes involved in ethane, propane and butane metabolism in bacteria. Present investigation was undertaken to fill the gap of our understanding of biochemical and genetic aspects of butane utilization by bacteria.

Studies were done on *Pseudomonas* sp. IMT37 and *Pseudomonas* sp. IMT40, both of which could utilize butane as a sole source of carbon and energy. Butane monooxygenase (BMO), the key enzyme in the utilization of butane, which converts butane to butanol was studied in the whole cells as well as in the crude extract. BMO in the whole cells was found to be an inducible enzyme. Assay was based on epoxidation of butene to epoxybutane, which could be quantitatively detected by a Gas chromatograph (Porapak Q colunm). One hour of reaction time and 50mg/ml cell density was found to be optimum for the assay. BMO activity ranged from 18-25 μmoles/g cells/hr. Butane grown cells of both the organisms, showed propane monooxygenase and hexane monooxygenase activities indicating a broad substrate specificity of the enzyme. Since the growth of these organisms was slow on butane, attempts were made to grow them on other carbon sources first and to induce BMO activity in the second step. Citrate was found to be the best carbon source for this purpose and BMO specific activity was found to be similar as that of cells grown solely on butane. Competitive inhibition of alkene epoxidation activity by butane and hexane in *Pseudomonas* sp. IMT37 ruled out the possibility of a separate alkene (butene monooxygenase) in the system. There was a proportional decline in the BMO specific activity as the butane or hexane concentrations were increased in the reaction vial. Specific activity was reduced to half of the original when butane concentration was increased to same level as that of butene. On the other hand, 1μl of hexane was sufficient to reduce the BMO specific activity to half of the original with its total abolition when 4μl of hexane was added in the reaction vial. Epoxides formed in whole cell assay, was found to be excreted out of the cells and was recovered in the reaction mixture supernatant. Stability of the enzyme was checked in the whole cells. Fifty percent activity was lost in the whole cells within 20 hours of storage at room temperature

and within 48 hrs at 4°C. Freezing and thawing of cells completely inhibited the BMO activity in the cells. For preparation of crude extract, cells were broken by French pressure cell operating at 28000 psi. Other methods for breaking the cells (sonication and lysozyme treatment) were not found suitable. BMO activity in the crude extract ranged from 80-125 nmoles/mg/hr. BMO specific activity of the crude enzyme was most stable at 4°C in 5% glycerol (half life-96 hrs). Without glycerol, it lost half of its activity in 24 hrs. Half-life of BMO could not be extended more even after trying various other stabilizing agents such as DTT, sodium thioglycolate etc.

Fractionation of crude extract by ultracentrifugation into membrane and cytosolic components was done and each component was checked for activity. Membrane or cytosolic fraction alone had no detectable BMO activity. However, activity could be reconstituted by mixing pellet (membrane fraction) and supernatant (cytosolic fraction). BMO activity could not be detected by mixing cytosolic fraction from non-induced cell with that of membrane fraction from induced cells or *vice-versa* but could only be detected on mixing the cytosolic fraction and the membrane fraction of induced cells, thereby indicating the inducible nature of the components of BMO. To identify the active components in membrane and in cytosolic fraction; exogenous addition of reductase, ferredoxin and rubredoxin in the assay mixture containing only membrane or cytosolic fraction was tested. BMO activity could be reconstituted when ferredoxin (spinach) and reductase were added to membrane fraction, suggesting that reductase and electron carrier component was cytosolic in nature and catalytic component (hydroxylase) was membrane bound. BMO was not inhibited by acetylene thereby suggesting that enzyme was different from the butane monooxygenase reported by Arp et al. (1999), who also showed diversity in BMO enzyme from different organisms.

SDS-PAGE pattern of induced versus non-induced cells showed induction of 42 kDa protein in butane exposed cells within 12 hours, suggesting that 42kDa protein may be involved in butane utilization. In order to find out if any additional protein was induced (even in minor amount) in butane induced cells radiolabelling studies were performed to identify only those polypeptides which are being formed at the time of labelling (pulse-labelling). Several other polypeptides (in addition to 42 kDa) were identified by ^{35}S methionine, which were present only in butane induced cells.

BMO activity was found to be in the void volume when the fractionation of crude extract was done by gel filtration on Sephacryl S-200 column. Only 3.2 fold purification could be achieved. In order to purify the active components in cytosolic fraction, membrane

fraction was separated by ultracentrifugation and cytosolic fraction was loaded on to Sephacryl S-200 column (30 cm x1.5 cm). Fractions containing active components were identified by mixing alternate fractions with the membrane fraction and checking reconstitution of BMO activity. Fractions in which BMO activity reconstituted still eluted in the void volume. Fold purification was around 4.3 fold in this case. All efforts to solubilize the membrane for further purification were unsuccessful. SDS-PAGE of crude extract, membrane fraction, cytosolic fraction and the pooled cytosolic fraction from Sephacryl S-200 suggested that a 42kDa protein was present in the cytosolic fraction and got enriched in fractions containing maximum reductase activity. Further, reductase assay using cytosolic fraction suggested that most of the activity was present in this fraction. Reductase component of alkane monooxygenase was reported to be 49 kDa (Ueda et al., 1972) and of methane monooxygenase was reported to be 39 kDa (Colby and Dalton, 1978). Thus there was a possibility that the 42kDa protein seen in induced cells and in cytosolic fraction (but not in membrane fraction) was the reductase component of BMO. This is the first report of characterization of BMO in the crude extract of bacteria.

In order to investigate the pathway of butane utilization, a number of mutants of *Pseudomonas* sp. IMT37 and IMT40 were isolated on the basis of their inability to grow on butane. These mutants could be grouped into at least three phenotypic classes. Representatives of each phenotypic group were checked for the presence of enzymes involved in the initial part of the pathway. Wild type had BMO activity (as described above), 1-butanol dehydrogenase activity, but no 2-butanol dehydrogenase activity. Investigation was carried out to detect the initial intermediate(s) in the utilization of butane. Mutants (83 and 91) of *Pseudomonas* sp. IMT 40 lacking 1-butanol dehydrogenase activity showed accumulation of 1-butanol. In these two mutants, BMO activity was similar to wild type. Comparison of retention time and co-elution experiments confirmed that the product was indeed 1-butanol and not 2-butanol. In other two mutants viz. 6 and 78 neither butane monooxygenase activity nor accumulation of 1-butanol could to detected. Wild type strains did not accumulate 1-butanol presumably because this transient intermediate was rapidly converted to next intermediates of the pathway. Data provided direct evidence for the conversion of butane to 1-butanol thereby allowing only terminal pathway to be followed in these strains.

Differential Display technique was used to identify DNA fragments involved in butane utilization by *Pseudomonas* IMT37. Total RNA from butane induced and non-induced cells were converted into cDNA using different primers and reverse transcriptase. The resulting

cDNA population was amplified by hot-PCR using *Taq* DNA polymerase and different arbitrary primers of which one was that used in cDNA preparation. Amplified products were run on a 6% acrylamide gel containing 50% urea. Autoradiography of the gel revealed the differences in the RNA expression patterns. Bands, which were only seen under induced conditions, were excised and reamplified (by normal PCR using the same set of primers) and used to probe the RNA blot; thus confirming that amplicons were really derived from induced RNA. By this technique, at least two DNA fragments were identified. Role of these fragments in butane utilization needs to be analyzed further.

Pulsed field gel electrophoresis (PFGE) was standardized for use in resolving *Pseudomonas* IMT37 genome and estimating the total genome size. *Spe*1 digest of genomic DNA could be resolved in 27 fragments whereas I-*Ceu*1digest could be resolved in 5 fragments. Total genome size was calculated to be around 4.75 Mb. A 4.9 kb DNA fragment, previously cloned in our laboratory was found to contain an ***orf*** encoding a 54 kDa protein, which was found to be essential for butane utilization (Padda et al., 2001) by *Pseudomonas* sp. IMT37. As a part of ongoing investigations on the genetics of this organism with special reference to butane utilization, the 4.9kb fragment was localized on the PFGE resolved genome.