4.1 BIOCHEMICAL STUDIES

Biochemical and genetic aspects of methane and liquid alkane utilization by bacteria have been quite extensively studied. In contrast, biochemical, physiological and genetic aspects of propane and butane utilization has not been studied in depth. Some basic work on biochemical aspects of propane and butane metabolism were available in the literature but these observations were not supported by genetic or molecular data.

This work was initiated to gain knowledge on the key enzyme of the pathway, butane monooxygenase (BMO) and to find out genetic basis of butane metabolism in bacteria. Many strains of bacteria were isolated from petroliferous region of Gujarat (India), of which two, *Pseudomonas* sp. IMT37 and *Pseudomonas* sp. IMT40, was selected as model organisms for studying utilization of butane. *Pseudomonas* sp. IMT37 was found to be a new species and was named as *Pseudomonas indica* sp. nov. (Type strain) and *Pseudomonas* sp. IMT40 was found to be a strain of *Pseudomonas* sp. IMT37 (Pandey et al., 2002). A 4.9 kb fragment encoding a 54 kDa protein (orf 54) was cloned by immunoscreening of the genomic library of the organism. This protein was found to play an essential role in butane utilization and also of other alkanes (Padda et al., 2001).

However, not much study was carried out on the biochemistry and genetics of the key enzyme propane and butane monooxygenase (PMO and BMO, respectively). The enzyme propane monooxygenase involved in propane utilization was reported to be very labile and difficult to assay in crude extract due to difficulty in breaking the cells (Woods and Murrell, 1989). As a result, the enzyme eluded purification and detailed biochemical and molecular information was lacking.

4.1.1 STUDY OF BMO IN WHOLE CELLS

Available reports on butane metabolism (van Ginkel et al., 1987; Phillips and Perry, 1974; Arp, 1999; Hamamura et al., 1999) till now were mainly concerned with the pathway of butane utilization, based on product accumulation and competitive inhibition with little or no emphasis on the key enzyme butane monooxygenase (BMO). van Ginkel et al. (1987) reported for the first time the substrate dependent oxygen uptake by *Nocardia* TBI grown on various substrates and specific activity was 53 nmoles/mg/min for butane grown cells (endogenous oxygen uptake being 12 units). Activity was observed only when molecular
oxygen was included in the reaction mixture, indicating that a monooxygenase was probably responsible for the oxidation of the hydrocarbon. Later on, Hamamura et al. (1999) reported the measurement of butane oxidation (as a measure of BMO activity) rate by measuring the disappearance of butane in butane incubated cells in an assay mixture (20-40 n mole/mg protein/min). While the oxygen uptake method was complicated by endogenous respiration of cells, the latter method of measurement of butane degradation by measuring the disappearance of butane suffered from the disadvantage of complicated steps involved in the assay, for example buffer saturation by butane and oxygen. In the present investigation, the method for assay of butane was adopted with slight modification from Ashraf and Murrell (1990), which was originally prescribed for propane monooxygenase. The procedure relied on the quantification of respective epoxides (1,2 epoxypropane, 1,2 epoxibutane, and 1,2 epoxyhexane) formed when the butane grown cells were supplied with propene, 1-butene or 1-hexene in the assay mixture. This method was also used for measuring alkane monooxygenase activity (van der Linden, 1971). In order to know the time course for epoxide formation during the reaction, 10 μl of the reaction mixture was injected into the GC after 10, 20, 30, 40, 50, 60 and 120 minutes, and epoxide formed was quantified. Table 3.5 represents BMO specific activity at different time intervals. This is also depicted graphically (Fig. 3.1). Though the specific activity does not vary much from 10 to 60 minutes, the total activity is very less in the initial phases of the reaction. Thus, reaction time of 60 minutes was chosen for the assay because this would be helpful in detecting even low level of activity in multistep process like purification of enzyme.

As a part of optimizing the assay system, BMO specific activity was also checked for different cell densities in the reaction mixture viz. 10, 20, 30, 40, 50 and 100 mg/ml. The specific activity values corresponding to each of these densities have been represented in Table 3.6. It is apparent that specific activity was highest when the cell density was 50 mg/ml. Therefore this density was used for all the experiments.

Butane monooxygenase activity in whole cells grown on butane was between 18-25 μ moles/g cells/hr. Assuming 12 mg of total protein was obtained from 1g of cells (Section 3.1.2), the value of BMO activity for whole cells could be translated into 25-33 n moles/mg proteins/min, a value quite comparable to the one (20-40 units) reported by Hamamura et al. (1999). Specific activities of this enzyme using propene, butene or hexene as substrate were calculated for both the organisms grown on butane or hexane as sole source of carbon. Since
the enzyme could also oxidize other substrates like propene and hexene, it behaved like PMO and HMO in addition to being a BMO. Similarly cells grown on hexane also showed BMO, HMO and PMO activities. The term PMO/BMO/HMO has been used in this section to indicate the above property of the enzyme. The PMO/BMO/HMO specific activity values for both the organisms have been represented in the Table 3.1. It is evident from the table that BMO and HMO activities were comparable in the individual strains but that affinity of the enzyme for propene was lower than that of butene or hexene indicating possible similar behaviour of the enzyme towards respective alkanes.

No BMO activity was detectable in cells grown on other carbon sources like glucose, succinate or citrate indicating that butane monooxygenase was not constitutively expressed. However, BMO activity could be detected in butane exposed cells after initial growth on citrate, glucose and succinate. The level of BMO induction in such cases was similar to that of BMO level in cells grown solely on butane. This clearly showed the inducible nature of BMO. Although these two organisms could not grow on propane, the enzyme could oxidize propene (and very likely propane) albeit with lower affinity, indicating its broad substrate specificity.

Whether the enzyme converted butane to form 1-butanol (terminal pathway or 2-butanol (subterminal pathway) or both was not clear. Because of transient nature, these intermediates could not be detected by GC and therefore, epoxide formation (which were not further metabolized) was a better measurement of BMO activity. Since alkenes (butene, hexene etc) were used as substrates for determination of BMO activity it was necessary to rule out the existence of separate butene monooxygenase in these two organisms. In order to prove that butane monooxygenase was the only enzyme converting butene to epoxybutane in the cells, different amounts of butane/hexane were added to the reaction vial. Epoxybutane formation was inhibited increasingly with the increase of butane in the reaction vial. Maximum inhibition was achieved when butene and butane were in the ratio of 50:50(v/v). This clearly showed that butane and butene were competing for the same active site. Such a competitive inhibition of PMO by propene was also reported by Woods and Murrell (1989). Even addition of hexane had a drastic effect on epoxybutane formation suggesting that the hexane also competed for the same site (Table 3.8). Above observations clearly suggested that the enzyme induced was a butane monooxygenase and that the enzyme induced in the organism by exposure to butane and hexane were the same having broad substrate specificity.
Epoxides are known to be toxic to cells. It was thus imperative to check whether the product, is retained inside the cells or exuded out into the supernatant. For this, the BMO activity was checked in supernatant as well as in the pellet after centrifuging the reaction mixture. The pellet was washed in the buffer (20mM Tris.Cl, pH 7.2) and resuspended in the same volume of buffer. Resuspended cells (10 μl) and 10 μl of supernatant was injected into the GC and the amount of epoxybutane was quantified. All the epoxybutane formed was found in supernatant thus indicating that all the epoxides formed was exuded out of cells.

In preparation for purification of the enzyme, it was important to know the half-life in whole cells. Half-life and stability under different storage temperature was evaluated in the whole cells of *Pseudomonas* sp. IMT37 (Table 3.7). BMO lost half of its original activity (24.32 μmoles/g/ hr) within 20 hours of storage at room temperature, and within 48 hours, at 4°C. When stored at −20°C for 8 hrs, the BMO activity was not detectable. This indicated that the enzyme was sensitive to freezing and thawing. Therefore, the best storage temperature for the enzyme was found to be 4°C.

### 4.1.2 CHARACTERIZATION OF BMO IN THE CRUDE EXTRACT

The biomass production was found to be very slow and poor (OD<sub>600</sub> = 0.25) even after 48 hrs, when the cells were grown in minimal media with butane. As a result, planning for detailed study of the enzyme was difficult. Therefore, other carbon sources such as, pyruvate, citrate, succinate, glucose and LB was tried to generate biomass in the first step and induce the enzyme in the second stage. Although, growth was maximum in LB (OD<sub>600</sub> = 0.867) followed by citrate (OD<sub>600</sub> = 0.6) the BMO specific activity was less than half compared to butane grown cells. Although the specific activity in pyruvate, glucose and citrate grown (and butane induced) cells were comparable, only citrate supported maximum growth. Citrate was therefore chosen as the best carbon source for growth and then exposure to butane for induction of monoxygenase activity. Cells grown in citrate for 6hrs (O.D<sub>600</sub> ~ 0.5) and induction with butane for 21 to 32 hrs was found to be most optimum (Table 3.2).

Till now there was no report on BMO activity in crude extracts of butane utilizing bacteria although Woods and Murrell (1989) demonstrated PMO in the crude extract. Purification of PMO was hampered because propane grown cells were difficult to break due to tough cell wall and also the enzyme was found to be highly unstable (half-life 150 minutes)
(Wood and Murrell, 1989). This might be the case for BMO as well. It was thus imperative to optimize the condition for breakage of cells and stability of BMO in the crude extract of *Pseudomonas* sp. IMT37. For breakage of cells three methods were tried; enzymatic lysis using lysozyme, homogenization and French press. Treatment of cells with lysozyme followed by osmotic shock was not satisfactory because it yielded only 3 mg/ml in the crude extract which was much less compared to French press method in which case 12.5 mg/ml protein concentration could be achieved. Homogenization resulted in 5.2 mg/ml protein, which was still not as good as French press method. Poor lysis by lysozyme and homogenization might be due to tough cell wall structure of hydrocarbon grown cells. This was strengthened by the fact that French press operating at less than 28000 psi also resulted in poor lysis (data not shown). It was observed that in routine work where cells were grown on only on citrate, it was easier to lyse the cells (data not shown). Therefore, it could be concluded that growth in hydrocarbon makes the cell wall less amenable to breakage. Total activity (in 10 ml crude extract) was also highest in French press method indicating that enzyme activity was not lost even when pressure as high as 28000 psi was applied for the breakage of cell. Therefore, French press was chosen as the method for preparation of crude extracts from *Pseudomonas* sp. IMT37 cells grown on butane (Table 3.9).

Enzymes should be reasonably stable for experiments spanning over longer duration like purification etc. Stability of the enzyme in the crude extract was an important factor to take into consideration. In order to know how stable the enzyme was, aliquots of crude extract prepared as described above was stored at different temperatures (25°C, 4°C, and -20°C). BMO activity was checked in each of these samples at regular intervals. The fresh crude extract (0 hrs) had BMO specific activity of 114.2 nmoles/mg protein/hr. BMO specific activity in the samples stored at room temperature for 3 hrs lost about one-third of the original activity. No BMO activity was detectable in the sample stored at -20°C (after 3 hrs of storage) suggesting that enzyme was susceptible to freeze and thaw. BMO activity was however retained to 50% of the original in samples stored at 4°C. This suggested that the best storage temperature for BMO was 4°C (Table 3.11). In view of unstable nature of BMO in the crude extract, different agents were tested for improving stability of the BMO. Enzyme activity appeared to be adversely affected by EDTA, thereby indicating that metal ions might be essential for enzyme activity. Detergents, PMSF and NaCl also had a diminishing effect on the BMO the reason for which was not clear. Glycerol, however appeared to protect BMO
activity up to 24 hrs at levels similar to that of original crude extract (Table 3.12). Glycerol is known to stabilize the enzyme by reducing the water effect. Subsequently different concentrations of glycerol (5% to 25%) were checked. Similar results were obtained with all these concentrations and BMO half-life could be extended up to 96 hrs (data not shown). Thus, 5% glycerol was included henceforth, in the extraction buffer for maintaining BMO stability.

4.1.2.1 Partial purification of BMO by gel permeation chromatography.

Once the characteristics of BMO in the crude extract were defined and the activity was stabilized for reasonable period of time, it was imperative to purify it. Purified form of BMO could answer many questions relating to structure function relationship, similarities between known monooxygenases and ultimately could give way to the gene encoding it. With the aim to purify the BMO, crude extract was subjected to gel permeation chromatography through Sephacryl S-200 column. All the BMO activity was found to be in the void volume. Around 3.2 fold purification could be achieved. This was the first report of partial purification of BMO activity (Fig. 3.3 and Table 3.13).

4.1.2.2 Localization of BMO

Since all the BMO activity eluted in the void volume (as described above) it appeared that either there was a large scale aggregation or the enzyme was membrane bound.

In order to study the location of BMO, crude extract was subjected to ultra centrifugation (1,50000 x g for 2hrs at 4°C) to separate membrane and cytosolic fractions. BMO activity could not be detected in any of the fractions but was detectable when membrane fraction and cytosolic fraction were mixed in 1:1 ratio (v/v). Around 90% of the activity could be reconstituted. In order to find the best stoichiometric ratio of membrane and cytosol for reconstitution of BMO activity, different ratio of membrane and cytosol were mixed and BMO activity was checked in all the combinations. BMO activity was recovered 100% when membrane and cytosol fractions were mixed in a ratio of 1:3 (Table 3.14).

The reconstitution experiment clearly showed that BMO was a multicomponent enzyme and that components were distributed both in membrane as well as in cytosol and that
all the components were essential for BMO activity. Monoxygenases are known to consist of catalytic component i.e., hydroxylase, a reductase and a carrier protein. It is generally accepted that reductase and carrier protein may be non-specific in action to some extent as they may be constitutively produced and required in cells for functions other than their role in hydrocarbon oxidation. Hence, logically, it may seem possible that only the hydroxylase component is induced by exposure of cells to butane and other components are recruited from the cellular metabolic pool. To check whether all the three components were induced specifically in response to exposure to butane, reconstitution experiment was done to complement the membrane fraction of induced cells with that of the cytosolic fraction of non-induced cells and vice-versa. BMO activity could be restored only when membrane fractions and cytosolic fractions were both derived from induced cells. Other combinations (like membrane fractions from induced cells and cytosolic fractions from non-induced cells, membrane fractions from non-induced cells and cytosolic fractions from induced cells, both the fractions from non-induced cells) were not effective in reconstituting BMO activity (Table 3.15). Results suggested that both the cytosolic components as well as membrane components of BMO in Pseudomonas sp. IMT37 were specifically induced in response to butane.

In order to achieve higher level of purification it was decided to purify the active components of membrane and cytosol separately, and then reconstitute the enzyme by combining purified fractions. For this, gel permeation chromatography of cytosolic fraction, using sephacryl S-200 was performed and alternate fractions were mixed with membrane components. Here again, the active components of the cytosol were recovered in the void volume. This suggested that either the cytosolic components were large (more than 250 kDa M.W) or they have tendency to aggregate. However this approach resulted in 4.3 fold purification of the enzyme (Fig 3.7).

In order to have an idea of protein the protein profiles of membrane and cytosol from induced cells, SDS-PAGE of these components was done along with crude extract. Certain polypeptides like 63 kDa, 45 kDa, 42 kDa, 38 kDa and 24 kDa M.W were present in cytosolic fraction but not in the membrane fraction. Similarly, a 40 kDa polypeptide was present in the membrane fraction but not in the cytosolic fraction (Fig 3.8). It was difficult to identify from the protein profiles, components of BMO in membrane and cytosol. A more definite way was to compare the banding pattern of cytosolic fractions producing the activity
on reconstitution. In order to do so, SDS-PAGE of crude extract, membrane fraction, fraction 9 (fraction resulting in the highest BMO specific activity in reconstitution experiment) and the pooled fraction was performed. Analysis of the banding pattern showed that a polypeptide of about 42 kDa M.W was progressively getting enriched in pooled fraction as well as in fraction 9 compared to original cytosolic fraction (Fig 3.9). This 42 kDa polypeptide was not seen in non-induced cells (Fig. 3.4). Molecular weight of reductase component of other monooxygenases were reported to range from 30 kDa to 40 kDa (Colby and Dalton, 1978; Ueda et al., 1972). Because of similarity of molecular weight and of cytosolic location, it is tempting to speculate that this 42 kDa polypeptide could be the reductase component of BMO.

4.1.2.3 Study of the cytosolic component

In order to see if reductase activity was present in membrane fraction or in cytosol, assays were performed using these two fractions and crude extracts of induced cells. Reductases could be assayed independently by using potassium ferricyanide acting as artificial electron acceptor in a reaction mixture containing protein to be assayed and electron donor NADH (Miura and Dalton, 1995). Maximum activity (285 units) was found in pooled cytosolic fraction, whereas very less (45 units) could be detectable in membrane fraction. Crude extract and original cytosolic fraction had the reductase activity of 175 units and 202 units respectively. Most of the reductase activity thus appeared to be cytosolic. This observation suggested that cytosol probably contributed the reductase component but whether it was specific for BMO or not could not be concluded with certainty as reductases were known to be non-specific (Ueda and Coon, 1972). Reductase component of alkene monooxygenase from *Nocardia* and of a methane monooxygenase was purified and their specific activities were 424 units and 6000 units respectively (Miura and Dalton, 1999).

In order to further probe into the nature of membrane bound and cytosolic components, membrane fraction was assayed for BMO activity in presence of exogenously added electron carrier protein- ferredoxin (spinach or *Clostridium*) or rubredoxin and ferredoxin reductase (Section 3.1.2.8). Addition of ferredoxin (spinach) and ferredoxin reductase to the membrane fraction could produce a significant level (63 nmoles/mg protein/hr) BMO activity. ferredoxin or the reductase alone was not effective. In contrast,
addition of these two components to cytosolic fraction could not reconstitute BMO activity. As shown earlier, mixing of cytosolic and membrane fractions was needed to reconstitute BMO activity. Taking all these observations into consideration, it could be strongly suggested that cytosolic fraction contributed the carrier protein and the reductase components in the butane monooxygenase activity of the organism. It is therefore reasonable to deduce that the hydroxylase component of BMO is membrane bound like that of alkane monooxygenase (Ruettinger et al., 1977). Rubredoxin or Clostridium ferredoxin in combination with ferredoxin reductase could not reconstitute BMO activity suggesting that some specificity is required among these three components. Thus, the model of the BMO system appears to be more or less similar to that proposed for alkane hydroxylase. It differs from both the forms of methane monooxygenase i.e., pMMO and sMMO in which all the components of oxygenase are membrane bound (pMMO) or cytosolic (sMMO). However, lack of reconstitution of monooxygenase activity by hydrogen peroxide addition to membrane fraction, and lack of inhibition by acetylene (data not shown) contrasts it with alkane monooxygenase system (Jiang et al., 1993). Acetylene was reported to inhibit BMO in three strains studied by Hamamura et al. (1999) though the extent of inhibition varied widely between the strains. They reported the diversity in BMO from all these strains based on this and other properties. Thus, BMO from Pseudomonas sp. IMT37 appeared to be a novel multicomponent oxygenase system.

4.1.2.4 Solubilization of membrane

It was clear that an active component(s) of BMO was located in the membrane and there is strong indirect evidence that the component(s) could be a hydroxylase as shown in the previous section. In an attempt to purify the component(s) it was essential to solubilize the membrane without affecting the BMO activity. Different detergents were tried. It was presumed that detergents could form micelles around individual active component protecting it from potential aggregation (owing to hydrophobic, membranous character, which, then could have been purified. Detergents tested (Table 3.16) were not satisfactory enough to solubilize the membrane and at the same time retaining BMO activity intact. Sarkosyl and octyl glucoside were inhibitory to BMO activity in the crude extract at all the concentrations tried. Sodium deoxycholate and saponin was not inhibitory to BMO activity in crude extract but was not efficient in solubilizing the membrane fraction of butane induced cells of
Pseudomonas sp. IMT37. Solubilization upto 70% could be achieved by 0.2% SDS but even at this concentration of the detergent, it was inhibitory to BMO activity.

Detergents commonly used for solubilization of many membrane preparations were not effective in this case. This inability could possibly be attributed to changes in membrane fluidity and/or cell wall changes reported to be associated with exposure of cells to few alkanes (Kennedy and Finnerty, 1975). An effective solubilizing agent for membrane preparation of IMT37 still remains to be found.

4.1.3 INDUCTION OF BUTANE SPECIFIC POLYPEPTIDES

As has been discussed earlier BMO is an inducible enzyme. Therefore exposure of citrate grown cells to butane must result in the induction of new proteins in the cell. In order to identify the polypeptides induced in response to butane exposure, SDS-PAGE analysis of induced versus non-induced cells was done. This revealed a polypeptide of M.W 42 kDa present only in cells exposed to butane for 12 hrs or more (Fig. 3.4).

All the proteins present in cells are stained by Coomassie blue and differences between protein profiles of induced and non-induced cells might not be very apparent. Radiolabelling with $^{35}$S-methionine in induced and non-induced cells was, a better alternative in which case only polypeptide synthesized at the time of labeling (pulse-labeling) would be labeled and could be detected by autoradiography of SDS-PAGE gels. Cells were labelled after 3 hrs, 6 hrs, 12 hrs and 24 hrs of exposure to butane. Control sets in which only citrate was added as a carbon source was taken for labeling after 12 hrs and 24 hrs. Comparison of labeling patterns, between induced and non induced cells when cells were pulse labeled for 30 minutes revealed several major new polypeptides unique in butane induced cells viz. 52 kDa, 62 kDa, 66 kDa and 97 kDa. All these polypeptides were present in 3 hrs, 6 hrs, 12 hrs and 24 hrs induced cells (Fig 3.5). In addition to these, 36 kDa, 42 kDa, 60 kDa and 70 kDa polypeptide were only present in induced cells exposed to butane for 12 hrs or more. But when the cells were labeled for only 5 minutes a polypeptide of M.W ~ 42 kDa was found to be present only in induced cells (9 hrs, 12 hrs and 24 hrs exposure) (Fig 3.6). This reflected a more prominent role of this polypeptide in butane utilization. Since these polypeptides are only present in butane induced cells, they may play some role in butane utilization.
4.2 GENETIC STUDIES

4.2.1 Analysis of butane utilization pathway

Not much information is available on the biochemical pathway involved in the utilization of butane by bacteria. Earlier works (van Ginkel et al., 1987; Phillips and Perry, 1974; Arp, 1999) indicated a terminal pathway for butane utilization, but there was no genetic evidence for the same. These observations were mainly based on changes in the fatty acid profile (Phillips and Perry, 1974), simultaneous adaptation studies (van Ginkel et al, 1987) and competitive inhibition experiments (Arp, 1999). Availability of mutants defective at each of the step of the metabolic pathway might facilitate study of various aspects of metabolic pathway. This approach was used by Ashraf and Murrell (1992) to dissect propane utilization pathway. They however did not measure or characterize the accumulated product in mutants. In view of lack of direct evidence for the terminal or subterminal pathway (or both) of butane utilization, mutants defective in utilizing intermediates (butane, butanol etc) of the butane utilization pathway were generated and the accumulated product further analyzed.

Wild type *Pseudomonas* sp. IMT37 and IMT40 could grow on 1-butanol but not on 2-butanol. They showed about six fold increase in 1-butanol dehydrogenase activity on exposure to butane compared to unexposed cells (Table 3.20), indicating that 1-butanol dehydrogenase was an inducible enzyme. In contrast, 2-butanol dehydrogenase activity was hardly detectable in these two organisms even after exposure to butane. Lack of this enzyme might be the reason for its inability to grow on 2-butanol.

Mutants obtained by mutagenesis could be placed in three groups based on their phenotypes. It appeared that Group I mutants were defective in butane monooxygenase, Group II in butanol dehydrogenase because they were defective in utilizing butane and 1-butanol but could utilize intermediate downstream the pathway i.e., butyric acid. Defect in Group III mutants could not be pointed out but most likely it was further down the pathway (Table 3.19). A Group II mutant designated 6 (from *Pseudomonas* sp. IMT37) and three more mutants 83, 91 and 78 obtained from *Pseudomonas* sp. IMT40 (generously provided by Pandey, K K, 2001) using similar method was used for further biochemical analysis. These four mutants had very negligible 1-butanol dehydrogenase as well as 2-butanol dehydrogenase activity. Butane monooxygenase activity was intact in mutants 83 and 91 but not in mutants 6 and 78. Since butane monooxygenase activity was found to be intact in mutants 83 and 91 and since butanol dehydrogenase was defective in these two mutants, it
was assumed that if butane was supplied to these mutants, butanol would accumulate in the cells, which could be detected by GC. This hypothesis was proved right and butanol was indeed accumulated in these mutants (Table 3.21). Comparison of retention time and co-elution experiment suggested that retention time and co-elution coincided with 1-butanol and not with 2-butanol. All these findings led to the conclusion that butane utilization in these two organisms followed terminal oxidation pathway.

Vangnai and Arp (2001) also reported the involvement of 1-butanol dehydrogenase in butane oxidation by ‘Pseudomonas butanovora’. The 1-butanol dehydrogenase specific activity was reported to be highest in cells grown on butane (32.9 ± 1.7 nmoles/mg/min). The activities of extract grown on 1-butanol, 2-butanol or lactate were 10 ± 0.5, 4.4 ± 0.1 and 1.9 ±0.1 nmoles/mg/min. They however, did not measure the 2-butanol dehydrogenase activity in butane grown cells but they could show by activity staining of non-denaturing gels that 2-butanol dehydrogenase activity was much less than 1-butanol dehydrogenase in butane grown cells. Thus like Arp (1999) they also could not rule out the possibility of subterminal pathway to be operative albeit at a very low level in this organism.

4.2.2 Identification of transcripts by Differential Display

The technique of differential display has been used successfully to identify novel genes, which are differentially expressed under different environmental conditions. In bacteria mostly the technique has been used for identifying stress related genes (Wong and McClelland, 1994; Gill et al., 1999). It is a powerful tool to identify genes for which proteins have not been purified or known. Therefore, it could well be utilized in identifying butane specific transcripts when Pseudomonas sp. IMT37 is exposed to butane. In order to do so, total RNA from citrate grown cells (non-induced, control) and butane exposed cells (induced) were isolated and converted into cDNA (using primers and reverse transcriptase). The resulting cDNA population was amplified by hot-PCR (using two primers one of which was used for cDNA preparation)) and run on 6% urea 50% acrylamide gel. Autoradiography of the gel revealed cDNA bands unique to induced cells. These bands were cut and reamplified by normal PCR under similar conditions. Total of 5 bands (B1, 3a, 3b, 4a and 4b) were identified (Fig 3.10, 3.11) but subsequently only two these, B1 and 4b could be reamplified indicating that other bands might be spurious product which is usually encountered in this technique. B1 and 4b bands were radiolabelled to probe RNA blots (Northern). Positive signals were obtained in both the cases, thus confirming that these bands were indeed
specifically induced on exposure of cells to butane (Fig 3.12 and 3.13). These two transcripts produced on induction by butane were of different sizes but both were smaller than 1.5 kb as evidenced by their mobility below the 16S rRNA band. Presence of these transcripts in only butane-induced cells indicated that corresponding DNA segments could play some role in butane utilization by this organism. Further analysis however needs to be done to confirm this. These DNA fragments can be used as probes to fish out corresponding genes which can then be analyzed at sequence level.

4.2.3 Pulsed field gel electrophoretic analysis of _Pseudomonas_ sp. IMT37 genome

Pulsed field gel electrophoresis has been used successfully in a number of bacteria for estimation of total genome size, fingerprinting and mapping of genes on the genome such as in _Pseudomonas_ (Holloway et al., 1994; Romling and Tummler, 1994), _Xanthomonas_ (Chan and Goodwin, 1999) and _Vibrio cholerae_ (Mazumder et al., 1996). By using similar approach attempts were made here to estimate the genome size of _Pseudomonas_ IMT37 and also to map a butane specific gene. This information was of interest because this organism has now been described as a new species _Pseudomonas_ indica sp. nov. (Pandey et al., 2002).

DNA blocks were prepared and subjected to PFGE under different parameters (pulsetime, duration and voltage). The DNA was of very high molecular weight, as the DNA did not move out of the well. Absence of any smear in the gel suggested that the quality of DNA preparation was good. Even under different conditions of PFGE there was no indication of any other band in the gel. This suggested that the organism did not harbor a plasmid or any other high molecular weight replicon. Subsequently different rarecutters like Not1, I-Sce1, Swa1, Spe1, I-Ceu1 and Shf1 were used for digesting the genomic DNA. Digested DNA was run using different parameters for the separation of fragments. The genome might have one or no site at all for Not1, I-Sce1, Swa1 and Shf1 because even after treatment of the genome with these enzymes no other band was visible in the gel. Only Spe1 and I-Ceu1 digested DNA could be resolved into multiple bands. For resolving Spe1 digested DNA 10 sec pulse for 4 hrs, 25 sec pulse for 4 hrs and 50 sec pulse for 14 hrs (at constant voltage of 175) was found
to be the optimum. PFGE performed under these parameters resolved *Pseudomonas* sp. IMT37 genome into 27 fragments (*Fig. 3.14 and Table 3.22*). The best parameters for I-Ceu1 digested DNA separation was however, 70 sec pulse for 12 hrs followed by 160 sec pulse for 13 hrs at constant voltage of 175 in which case five distinct6 bands could be seen (*Fig 3.15 and Table 3.23*). Total genome size was calculated by adding the molecular weight of each fragment. Genome size estimated by summing molecular weights of restriction bands of Spe1 digested as well as I-Ceu1 digested were in close agreement and was around 4.75 Mb. Thus *Pseudomonas* sp. IMT37 genome size differed with genome of *Pseudomonas aeruginosa* strain PAO I, which was estimated to be 5.9 Mb (Romling et al. 1989). It might be possible that some overlapping bands could not have been separated in the present study despite exhaustive optimization of PFGE parameters. This might be the reason for underestimation of the total genome size.

I-Ceu1 cleaves DNA at no sites other than *rrn* operons. Thus it appeared that *Pseudomonas* sp. IMT37 has 5 *rrn* operons. However, I-Ceu1 has been reported to have 7 sites in many Gram-negative bacteria (and thus 7 *rrn* operons) for example *E. coli*, *Salmonella* (Liu et al., 1993) and *Vibrio cholerae* (Mazumder et al., 1995). Thus the observation that *Pseudomonas* sp. IMT37 has 5 *rrn* operons appears to contradict the hypothesis that the number of *rrn* operon in Gram-negative bacteria are conserved.

Once the PFGE genome fingerprint (for both Spe1 and I-Ceu1 digested DNA) of *Pseudomonas* sp. IMT37 was available, the next step was to localize the 4.9 kb fragment on the genome. For this, 4.9 kb fragment was radiolabelled by random priming and used to probe the PFGE blots (both Spe1 digested and I-Ceu1 digested). Hybridization and autoradiography revealed that 4.9 kb fragment hybridized with S10 fragment (of Spe1 digestion; *Fig 3.16*) and C3 fragment (of I-Ceu1 digestion; *Fig. 3.17*).