Discussion
5. DISCUSSION

5.1 Optimization of medium and environment for increased ALS and 2,3-BD production

In the recent years, 2,3-BD has received significant interest not only due to its status as an industrially important compound but also because its production is inhibited very less by the end product compared to other solvent fermentations (Fond et al., 1985). It is thus possible to accumulate high concentrations of butanediol in the fermentation broth by changing the various physical as well as chemical parameters. Generally BD is produced in late stationary phases of growth i.e. from 24 to 72 h (Zahler, 1976 and Renna et al., 1993) and this makes the whole process less economic. Hence, in the present study, the interest was to produce maximum BD in the early phases of growth by optimization of medium, environmental conditions, addition of chemicals and mutagenesis for the early induction of α-ALS (an enzyme involved in 2,3-BD pathway).

The entire process of fermentation depends on the substrate used and its suitability as growth and energy source. Out of carbon sources (glucose and sucrose) used in the present work, glucose was more efficiently used (Table 1 & 2) as it yielded high BD and ALS.

High substrate concentrations inhibited the production of BD and ALS in accordance with other reports (Laube et al., 1984a, Fages et al., 1985 and Magee and Kosaric, 1987) which can be explained by the dependence of microbial growth rate on the concentration of solute. Another reason
attributed to the inhibition at higher concentrations of substrate is the “crabtree effect” which denotes the repression of normal respiration by sugars probably by inhibiting the oxidative phosphorylation site I in the electron transport chain (Doelle, 1981; Payton and Haddock, 1985).

On the other hand, many authors have reported high yield of BD by using high concentrations of substrate in contrast to our study (Jansen et al., 1984; Sablayrolles and Goma, 1984). These variations in the results might be related to the organism and different fermentation conditions eg. aeration, nitrogen source, stimulatory contents and temperature applied in a particular procedure (Garg and Jain, 1995). Inhibition of the cell mass production, besides BD and ALS, was reported at the high concentrations of the substrate (glucose). In contrast to it, cell mass increased even when the optimum level of sugar (sucrose) concentration became doubled. The results are compared with the results of Chuang and Collins (1968), where these authors showed that the cell mass should increase with an increase in the substrate concentrations due to the enhanced synthesis of pyruvate.

In the present study, higher amount of yeast extract (3%) was required in basic medium to produce high amounts of BD (23.64 and 40.30 g/100g glucose utilized) and α-ALS (27.0 and 42.0 U ml⁻¹) after the 10 and 24 h of growth. These results could be attributed to the fact shown by Laube et al. (1984) in which higher concentration of glucose (5%) has been reported to require higher level of yeast extract (1.5%) for utilization in comparison to 0.3% yeast extract needed for 1% glucose. Lower concentration of yeast extract
lowered the cell growth and consequently BD and ALS production (Table 3), which is attributed to the fact that the nitrogenous nature of the yeast extract is essential for enhancement of protein synthesis (O’Kane, 1950). Moreover yeast extract also simulated the BD production (Ledingham and Neish, 1954 and Laube et al., 1984). Higher concentration of yeast extract lowered the BD production which could be due to the accumulation of the metal ions eg. Ca++, Cu++, Mo++, Zn++, Al+++ and phosphate which are the integral part of yeast extract (Laube et al., 1984).

Peptone was found to be less inhibitory to cell mass, BD and ALS production. Peptone in the medium enhances respiratory activity of the cell and decreases the cell death. Besides the respiration effect, it prevents the reduction of pyruvate to lactate (Finn, 1954 and Bruhn and Collins, 1970). The amount of peptone required (0.5%) was very less in basic medium to get the maximum yield of BD (17.72 and 36.0 g/100 glucose utilized) and ALS (22.2 and 33.0 U ml⁻¹) after 10 and 24 h of growth (Table 4).

Sodium pyruvate stimulated the production of BD and ALS (Table 5) at low (0.15%) concentration. But at higher concentration (>0.15%), decrease in the BD and ALS activity was reported. Pyruvate is an intermediate of glucose catabolism and it stimulates the production of BD and ALS (Jansen et al., 1984a). Generally pyruvate penetrates the cells and induces the production of acetolactate synthase (ALS) and acetolactate decarboxylase (ALDC) required for butanediol production (Speckman and Collins, 1973 and deCardenas et al., 1980) thus diverting the pathway towards BD production. In this study,
results showed concentration-dependent production. The increased concentration of sodium pyruvate inhibited the production. This fact may show that excessive pyruvate could cause the internal toxicity by automatic inhibition of pyruvate formation from glucose by glycolysis pathway and thus affecting the enzymes (α-ALS, α-ALDC and AR) production. In contrast to it, Hill et al. (1953) showed that excess pyruvic acid inside the cell enhanced the production of acetoin. Conversion of pyruvate to lactate or acetoin depends upon the organism used (Bussee and Kandler, 1961).

Initial pH of the medium changes the fermentation profile of 2,3-BD and mixed acids pathways. The pH 5.8 was found to be optimum for BD production and these results were in agreement with Grover et al. (1990) and Raspoet et al. (1991) where pH range of 6.0-6.2 was optimum for BD production. In our study, higher pH lowered the BD and ALS production. Neutral and alkaline pH (8.0) enhances the mixed acids production (Magee and Kosaric, 1987). At pH 5.8-6.0 acetalactate synthase enzyme is induced which increases the pyruvate transport, compensates for ATP and NADH and maintains the pH homeostasis (deMas et al., 1988; McFall and Montville, 1989 and Tsau et al., 1992).

Optimum temperature for BD and ALS production by E.cloacae was found to be 30°C (Table 8 & 9), though growth was best at 35-37°C. Generally optimum temperature for BD production varies from organism to organism. Klebsiella pneumoniae reduced the production of BD when temperature
increased from 33 to 37°C but this temperature hardly affected the BD production when Enterobacter aerogenes was employed (Barrett et al., 1983).

Change in supply of oxygen by altering the agitation speed or medium: flask volume ratio at various levels results in the increase or decrease of production of BD and AMC. The experiments performed with different volumes of medium and at different rpm showed that at 1:2.5 medium : flask volume ratio and at 180 rpm BD production was enhanced. BD and acetoin formation is O₂ dependent which showed the status of medium (substrate) and end product formation (BD) as a function of O₂ supply. Hence shake flasks are probably pre-requisite for scaling up to fermenter level (Groit et al., 1986). On the other hand Byun et al (1993) showed that inhomogenous supply of O₂ at optimum circulation with time gives maximum production of 2,3-BD.

Vitamins are vital for all forms of life and some organisms cannot synthesize them. Therefore, they acquire them from exogenous sources. In our observation, vitamins like thiamine, riboflavin and ascorbic acid were found to be neutral or inhibitory for growth, BD and ALS production, although riboflavin has been reported to form a part of yeast extract which has a confirmed stimulatory role for BD production as revealed in the current study and also reported by Alam et al (1990). Beside vitamins, the other constituents of yeast extract may also have an important role to play (Speckman and Collins, 1973 and Magee and Kosaric, 1987).

Thiamine is necessary in the nutrition of some microorganisms. Thiamine occurs in the cells largely as its active co-enzyme form – thiamine
pyrophosphate (also called co-carboxylase), which catalyzes the reactions in the carbohydrate metabolism in which aldehyde groups are removed and/or transformed:

(i) decarboxylation and α-ketoacids and

(ii) the formation or degradation of 6.0-6.2-ketoles.

In these reactions the thiazol ring of thiamine pyrophosphate serves as transient carrier of a covalently bound ‘active’ aldehyde group. Mg** is also required as a cofactor. Thiamine pyrophosphate alone promotes non-enzymatic decarboxylation of pyruvate to yield aldehyde and CO₂. The hydrogen-atoms of thiazol ring ionises readily carbon atom of pyruvate to yield CO₂ and hydroxyethyl derivative of thiazol ring. The hydroxy ethyl group then undergoes hydrolysis to yield acetaldehyde, or become oxidized.

Riboflavin exists in the form of a coenzyme flavin adenine dinucleotide (FAD). The FAD functions as prothetic group of oxidation-reduction enzyme known as flavo-enzyme or flavo-proteins. These enzymes function in the oxidative degradation of pyruvate, fatty acid and amino acids.

Glucose

↓

Amino Acids

Pyruvate

Fatty Acid

Acetyl Co-A

Riboflavin did not show any effect on BD and ALS production. Ascorbic acid was found to be inhibitory for α-ALS and BD production. Ascorbic acid is
not present in the microorganisms, nor does it seem to be required. It is a strong reducing agent, readily losing hydrogen atoms to become dihydroascorbic acid, which also has vitamin C activity.

Glutamic acid increased the production of $\alpha$-ALS and BD in the log phase. The cause for this increase has not been known yet.

Valine, leucine and isoleucine (branched chain amino acids which show a common pathway for their synthesis and involve $\alpha$-ALS) decreased the cell growth, BD and ALS production by >50% after the 24 h of growth in GYEP and SYEP media. The results were supporting the study of Yadav et al (1977), who reported that valine, leucine and isoleucine (1-10 mM) inhibited the cell growth and acetoin formation by $E$.cloacae 27613, $Torulopsis celliculosa$ NRRL 172 and $Saccharomyces cerevisiae$ NCYC 324. Yadav et al (1977) emphasised that valine, leucine and isoleucine do not inhibit acetoin formation by all the organisms ($Saccharomyces caribergensis, S. rouxi, S. veronae$ and $Schizosaccharomyces pombe$) studied and that valine offers feedback control of $\alpha$-acetolactate might be true for some organisms only.

Individually cysteine could not lead to the stimulation but rather inhibited the $\alpha$-ALS and BD production in GYEP and SYEP media. But as a part of yeast extract it helped in the induction of $\alpha$-ALS and BD production (Laube et al., 1984b).

Co-enzymes viz. FAD, NADP and NADPH showed inhibitory effect toward $\alpha$-ALS and BD production in $E$.cloacae. FAD generally acts as a
prosthetic group of oxidation-reduction enzymes like flavoenzymes. Animal
tissues and micro-organisms contain enzymes that catalyse:

\[
\text{NADPH} + \text{NAD}^+ \underset{\text{NADP}^+ + \text{NADH}}{\rightleftharpoons}
\]

The reaction permits utilization of reducing equivalents of NADPH by
respiratory chain, which normally accept electrons from NADPH as immediate
donor. In the reverse direction it allows the reduction of NADP^+ for
biosynthetic purposes. NADPH plays an important role as a reductant in
biosynthetic reactions.

Addition of organic acids like acetic acid, isobutyric acid and lactic acid
were found to stimulate the production of BD and \( \alpha \)-ALS. Acetic acid (0.02\%)
induced the BD production (by 64.0 and 55.0\%) and \( \alpha \)-ALS (by 76.0 and
65.0\%) after the 10 and 24 h of growth respectively as compared to the
unsupplemented GYEP optimised medium, while in SYEP medium BD was
induced by 41.0\% and \( \alpha \)-ALS by 48.0\% after 24 h of growth. Similar results
with low concentrations of acetic acid on its stimulatory role in the BD
production have been reported by Magee and Kosaric (1987). At higher
concentrations, our results showed progressive inhibition of BD and ALS as
also reported by Zeng et al (1990b). These facts could be explained on the basis
of following factors:

(i) Stimulation (at low concentration) of enzymes involved in BD pathways
    (Stromer, 1968b; Holtzclaw and Chapman, 1975).

(ii) Acetic acid can act as carbon source for acetyl-Co-A synthesis (Johnsen
     et al., 1975 and Willets, 1984).
(iii) Increase in the carbohydrate utilization (Barrett et al., 1983).
(iv) Conversion of acetate to BD by direct incorporation and condensation with active pyruvate (Magee and Kosaric, 1987).
(v) Shifting the metabolic pathway towards 2,3-BD production rather than mixed acid formation in response to the low pH formed by acetate (Maddox, 1988).

Appropriate concentration of acetic acid for the maximum production of BD has been found to be different with different organisms and environmental parameters. Yu and Saddler (1982b) reported that 0.1 to 0.5% concentration was optimum for maximum BD production and more than 1.0% concentration inhibited the production. Also 0.5% to 1.0% concentration was effective for BD production investigated by Laube et al (1984a). But Fond et al (1985) and Zeng et al (1991) reported strong inhibition at 0.5% and 1.0% concentrations. These results supported the results obtained in our study. Besides BD and ALS, cell growth was also found to be inhibited at higher concentration in our study as also reported by Yu and Saddler (1982a), Barret et al (1983), Bajpai and Iannotti (1988) and Tseng and Montville (1993).

Isobutyric acid is similar to acetic acid except that it has four carbon-chained structure. At lower concentrations (0.02%) it induced/increased the production of BD (by 58.0%) and ALS (by 64.0%) with increase in cell mass (18.38 g L⁻¹) in the GYEP medium in the log phase. While in SYEP medium BD and ALS was increased (by 47 and 45%) after the 24 h of growth. But at higher concentrations (>0.02%), isobutyric acid was found to be inhibitory for
cell mass, BD and α-ALS production like acetic acid. Hence isobutyric acid was found to be equally good at 0.02% concentration as compared to acetic acid for the induction of α-ALS and BD production in the current study. Our observation supported the fact that analogs of acetate (CH₃COO⁻) substituted at the methyl group produced similar results. Therefore, it appears that the functional group responsible for the inducing effect is the carboxyl group (COO⁻) of the ionised acetate (Magee and Kosaric, 1987).

Lactic acid has been reported to play an important role in the BD and α-ALS production. In the present study, 0.2% lactic acid in the medium (pH maintained at 5.8) was able to enhance the production of α-ALS and BD with little increase in the cell mass. Lactic acid is utilized by microorganisms as a carbon source in the later stages of growth for cell maintainance (Magee and Kosaric, 1987). But on the other hand, lactic acid helped in the induction of enzymes (eg. α-ALS) involved in the BD pathway, as found in our study and also described by Qureshi and Cheryan (1989). Moreover, lactic acid in the medium might favour the feed back mechanism by inhibiting the production of enzyme lactate dehydrogenase, which is involved in the direct conversion of pyruvate into lactate. At higher concentration (>0.2%) lactic acid inhibited the production of α-ALS and BD. It was only due to the formation of high acidity which could inactivate the enzymes involved in BD synthesis (deCardenas et al., 1985). Other organic acid like citric acid and oxalic acids were tried and were found to inhibit the growth, BD and ALS production in the medium (initial pH was maintained as 5.8). These results showed that
these acids themselves might be inhibitory for the growth and the production of BD and ALS of E. cloacae.

Lastly, the formation of a medium which combined all the optimised media/environment parameters was used to produce increased amount of BD (by 57.0 and 55.0%) and α-ALS (by 80.0 and 77.0%), at 10 and 24 h of growth respectively, in GYEP medium as compared to the basic medium. Production of BD was enhanced by 21.0 & 37.0% and α-ALS by 39.0 & 40.0% in SYEP medium as compared to the basic medium.

5.2 Effect of fatty acid salts

Production of 2,3-butanediol in GYEP (55-58 g/100 g glucose utilized) and in SYEP (51 g/100 g sucrose utilized) optimised media, shown in Fig.2 and 3, was maximum at 24 and 48 h of growth (stationary phase). This is because the enzymes produced in the 2,3-BD pathway are generally induced in the stationary phase (Zahler et al., 1976 and Renna et al., 1993). BD yields were found to be more than theoretical values (>50 g/100 g glucose or sucrose utilized). This may be because of the presence of yeast extract, peptone, pyruvate, lactic acid and acetic acid. These all ingredients may help in the activation of enzymes to enhance the production of 2,3-BD (Laube et al., 1984; Qureshi and Cheryan, 1989 and Zeng et al., 1990). The BD was produced maximally at 48th h of growth which makes the process time consuming and expensive. Since the first enzyme, α-acetolactate synthase, in the BD pathway plays an important role for the conversion of pyruvate (formed from glucose)
into acetolactate, so it was thought wise to induce the α-ALS in the log phase of growth to get early production of BD.

Acetic acid has been known to induce the enzyme α-ALS for BD or acetoin production (Mickelson and Werkman, 1938 and Happold and Spencer, 1952). These authors also found that propionic acid also stimulated the production of BD. In 1968, Stormer found that acetic acid helped in the induction of all enzymes involved in 2,3-BD pathways to help in the formation of butanediol from pyruvate. He used acetamide instead of acetic acid for the enzyme induction, but no stimulation or induction was observed. Hence it was concluded that the carboxyl group was essential for the stimulation of BD production. In the later studies, Stormer (1975) again showed that acetate (CH₃COO⁻) was responsible for the induction of acetolactate synthase of *K. pneumoniae*. He also found that similar results were produced when the analogs of CH₃COO⁻ substituted at the methyl group were used. Therefore it appears from their studies that functional groups responsible for the inducing effect is the COO⁻ of the ionised acetate.

In our study, we used of various organic fatty acids for induction of ALS. Out of these, acetic acid and isobutyric acid were found to induce ALS and stimulate the early production of BD (at the 10th h of growth). But these salts were effective at 0.02% concentration. At higher concentration >0.02% they tend to decrease the cell growth, production of ALS and BD drastically at the 10 and 24 h of growth. These results were similar to Frazier and Westhoff (1994), who showed the toxic effect of high concentration of acetic acids which
could cause the reduction of glucose fermentation (Maeson and Lako, 1952) by interfering with the electron transport system. Influence of acetic acid upon dissimilation of glucose stimulates the butanediol production coupled with decline in hydrogen evolution upon the addition of acid (Mickelson and Werkman, 1938). As these acids (acetic acid, propionic acids, butyric acids etc.) are weak acids and less dissociated in the solution and might enhance the acidity of the medium, they probably decrease the ALS and BD production in the medium at higher concentrations. Hence the acetic acid and isobutyric acid in GYEP and SYEP media were replaced with their respective sodium salts in order to get the CH₃COO⁻ ion or carboxyl group in more dissociated form to induce the ALS and stimulate BD production in the log phase of organisms growth and to reduce the toxic effect of these short chained fatty acids. Various fatty acid salts viz. sodium acetate, sodium formate, sodium propionate, sodium butyrate, sodium isobutyrate and sodium valerate (0.1 to 0.4%) were tested for the induction of enzyme and BD production. Only sodium acetate and sodium isobutyrate at 0.1% concentration increased the BD production by 63.72% (44.25 gm/100 g glucose utilized) and 6.56% (28.8g/100 g glucose utilized) at the 10 h of growth. Specific activity increased by 46% (20.51 U mg⁻¹ protein) and 107.2% (40.63 U mg⁻¹ protein) with sodium acetate and sodium isobutyrate in the log phase. The results were comparable with Holtzclaw and Chapman (1975) where specific activity was 91.2 U mg⁻¹ protein and 175.6 U mg⁻¹ protein in case of sodium acetate and sodium isobutyrate to get more production of acetoin from Bacillus subtilis. But in
our study, though we have more production of ALS by isobutyrate compared to the other fatty acid salts, we did not have enough amount of BD produced in the log phase. There was only 6.56% increase as compared to GYEP control and 1.53 times less than the BD produced by using sodium acetate. Sodium acetate was also found to be effective in the stationary phase to get maximum BD production (62.5 g/100 g glucose utilized) in the 24 h of fermentation.

In our study 0.1% concentration of the fatty acid salts were found to be optimum for maximum induction of ALS and production of BD in the log phase as well as late stationary phases. Our findings are similar to the Willetts (1984). He showed that the addition of relatively low concentrations of sodium acetate (<1.0 g L⁻¹) to the starch based growth medium caused substantial increase in the diol production and the addition of 5.0 g L⁻¹ sodium acetate caused severe growth inhibition and decreased the amount of BD produced.

According to Holtzclaw and Chapman (1975), 27 mM concentration of fatty acid salts were found to be optimum for maximum induction of ALS and BD production and most effective inducer was found to be isobutyrate i.e. twice as effective as acetate. In our study isobutyrate was found to be effective inducer for enzyme synthesis but it was not taken as fruitful medium supplement in order to get maximum BD production as compared to sodium acetate. Stormer (1968b) found that 150 mM of acetate (9.01 g L⁻¹) concentration was optimum to achieve maximum activity of all the three enzymes in 2,3-BD production.
Sodium propionate, sodium butyrate and sodium valerate were found to decline the growth and ALS activity as well as BD production gradually from 0.1 to 0.4% concentration. Propionate at lower concentration had weak inductive effect as shown by Holtzclaw and Chapman (1975). In contrast, propionate was found to elicit similar results as compared to acetic acid (Happold and Spencer, 1952 and Stormer 1968b). The following table showed ALS specific activity obtained by Holtzclaw and Chapman (1975) vs our studies by different fatty acid salts.

Effectiveness of various fatty acid salts on the acetolactate synthesis

<table>
<thead>
<tr>
<th>Medium supplement</th>
<th>Specific activity of ALS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>By Holtzclaw and Chapman (1975)</td>
</tr>
<tr>
<td>None</td>
<td>34.8</td>
</tr>
<tr>
<td>Acetate</td>
<td>91.2</td>
</tr>
<tr>
<td>Formate</td>
<td>-</td>
</tr>
<tr>
<td>Propionate</td>
<td>44.0</td>
</tr>
<tr>
<td>Butyrate</td>
<td>40.5</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>175.6</td>
</tr>
<tr>
<td>Valerate</td>
<td>25.0</td>
</tr>
</tbody>
</table>

Renna et al (1993) described that als R gene regulates the expression of acetolactate synthase gene als S and acetolactate decarboxylase gene als D. The role of Als R seems to be limited to increasing the transcription of als S in response to a signal that appears or accumulates in the stationary phase. They found that als R mutation results in the increased transcription through the als SD operon during both the exponential and stationary phases. Als S
activity in the strains lacking Als R is no longer inducible by acetate, suggesting that acetate induction may occur through Als R. It is possible that Als R activity influenced by change in intracellular pH or acetate or may be one of its derivatives is an inducing ligand of the Als R protein.

It is believed that Als R is present in the cells at all stages of growth, but in the absence of inducing ligand, it is not competent to activate transcription. Upon entry to the stationary phase, a signal appears or accumulates to some critical level and binds to Als R, initiating transcription of als S. This signal may be acetate or a related molecule.

Tripalmitin was also used in induction studies. It was noticed that cell mass was increased by 91.38% (at 10 h of growth) as compared to GYEP and GYEP containing different fatty acid salts media. These findings have lead to the use of various combinations of sodium acetate, sodium isobutyrate (potential inducers) with tripalmitin. But with these combinations, growth was not increased as compared to GYEP. But the media, (i) GYEP containing 0.05% sodium acetate and 0.05% sodium isobutyrate with 0.1% tripalmitin and (ii) GYEP containing 0.1% sodium acetate and 0.1% tripalmitin showed 50% increase in the BD production in the log phase. No previous study on tripalmitin with fatty acid salts in various combinations has been carried out yet.

Hence, in the present study, fatty acid salts viz. sodium acetate (individually or in combination with 0.1% tripalmitin), sodium isobutyrate (in combination with 0.05 to 0.1% sodium acetate and 0.1% tripalmitin) showed
high BD production (>45.0 and \(-40\) g/100 g glucose utilised) at 10 h and \((-62.5\) g/100 g glucose utilised) 24 h of growth respectively. BD production remained same till 48 h of growth. Hence 63% increase in the BD production was achieved in the log phase by fatty acid salts as compared to the GYEP medium.

Although different fatty acid salts at 0.1% concentrations were found to produce BD in different amounts in the log phase of growth. However actual proportional ratio between type of fatty acid salts (0.1%) used, the amount of BD produced and amount of ALS synthesized was found to vary with fatty acid salts. Hence it was concluded that ALS activity and specific activity was not found to be directly proportional to the production of butanediol by addition of different fatty acid salts (individually or in combinations). So there might be some reasons behind this fact. There may be:

(i) Induction and biosynthesis of other enzymes viz. ALDS (acetolactate decarboxylase) and AR (acetoin reductase) of 2,3-BD pathway (besides \(\alpha\)-ALS) are different by using different fatty acid salts.

(ii) Increase or decrease in the utilization of glucose at different level instead of inducing \(\alpha\)-ALS.

(iii) Activation of the different genes coding the enzymes involved in glycolysis and BD pathway expressing them at different levels to get the BD production.

(iv) Generally \(\alpha\)-ALS synthesis takes place in the stationary phase of growth. After that other enzymes are immediately synthesized. But the
fatty acids might transcribe the genes coding for all enzymes of BD pathway equally in the log phase.

5.3 Mutations

Optimization of culture medium and addition of various inducing/enhancing substances in the medium increase the yield of desired product to certain level. This approach will be limited by the organism's maximum ability to synthesize the respective product. Efficiency of the organism for the potential production of desired compound is controlled by the genes present in its genome. These genes must be modified to increase the yield of compound. Selection of mutants is one of the approaches. Mutagenesis by using ultraviolet radiations is the simple and convenient way to obtain wide variety of mutants. Kulia et al (1971) described the induction mutations by UV in Streptococcus for production of butanediol and related compounds. Phalip et al (1994) exposed L. lactis subsp. lactis biovar diacetylactis S, to UV radiations and obtained mutants that produced a mean of 4.5 times more acetoin and related compounds than the parental strain did. The UV mutations have been reported to be caused by formation of thymine-thymine dimer in DNA strand. Generally UV light is found to be effective mutagen for bacterial cells. Since the genetic material in these cells is not shielded by any great thickness of UV absorbing material (nuclear membrane), so UV helps in the formation of thymine dimers easily (Setlow and Carrier, 1966). Kulia et al (1971) suggested that exposure time corresponding to less than 1% survival was used to mutate the cells. But in our study, approximate 40% survival after
UV exposure was able to produce hypermutants in *E. cloacae*. Although in our study, more than 50% yield of 2,3-BD was obtained which is more than theoretical value obtained by using wild type *E. cloacae*. But the hypermutants, produced by UV, gave 1.4 times more yield of 2,3-BD (maximum 74-80 g/100 g glucose utilized) as compared to wild type *E. cloacae*. UV mutants, however, were not stable as after one month, significant decrease in the BD production was found (>2.0 folds). Because in UV induced mutations, lesions and thymine dimers in DNA are formed and could may different combinations of genes which might cause the reversion of mutants in short time interval. Hence UV mutants are not suitable for further use. Most mutants failed to show any enhancement in the BD production after log phase. This observation is a pointer towards the possibility that different sigma factors are involved which recognize and transcribe the genes of the BD operon in the log phase rather than stationary phase (Renna et al., 1993).

Ethyl methane sulfonate too, did not yield desired mutants. EMS primarily causes the transition mutations. On the other hand, mutations with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) were successful in yielding a stable mutant. In our study, following the treatment with NTG, one mutant of *E. cloacae* that produced 1.2 times more BD (compared to wild type) in the log phase (10 h) was isolated. The ALS activity in this mutant almost remained same (1.1 folds increase). This observation points to the fact that other enzymes viz. acetolactate decarboxylase and acetoin reductase might be induced instead of or besides ALS induction. α-Acetolactate decarboxylase
mutants were also obtained after mutagenesis with NTG by Monnet et al (1994). Boumerdassi et al (1997) showed that NTG mutants of *Lactis* subsp. *lactis* biovar diacetylactis CNRZ483 L2 & 483L3 converted almost 80% of glucose into butanediol, acetoin and diacetyl. On the other hand Platteeuw et al. (1995) showed that overexpression of α-acetolactate synthase in *Lactis* also leads to increased production of acetoin and 2,3-butanediol.

NTG induced mutations are effective in low lethal doses (Mandell, 1960; Adelberg, 1965 and Eisenstrack et al., 1965). There was remarkable decrease in the viable cell count, found in the stationary phase of bacterial cells when treated with NTG. But there was less decrease in the viable cell count, in the exponential phase (Cerda-Olmedo et al., 1967 and Yoshida & Yuki, 1968). These results were similar to the results obtained in our study. But this could not explain the reason as to why the cells at different phases of growth responded differently to the killing action of NTG.

In the present study, 54.2% survival of micro-organisms after 10 min was able to produce efficient mutant. These results were similar to those of Kulia et al (1971) who showed that 50% survival for *Streptococcus* with NTG after 10 min time was sufficient. There is no established theory of mutagenic action mechanism of NTG. It exerts its potent mutagenic action by different mechanisms depending upon the organism and their physiological state (Singer et al., 1968; Kasahara et al., 1971). Yoshida and Yuki (1968) could isolate less amount of DNA from *Bacillus subtilis*, treated with NTG. This might mean that NTG treatment breaks DNA molecules to the size
unprecipitable by ethanol. Mode of action, conditions and extent of mutation by any method of mutagenesis are variable from species to species and their physiological state.

However, the stable mutant obtained with NTG gave as much yield of BD after 10 h of growth as was given by the wild type (WT) after 24 h. So even though, mutant was not a hyper producer (only 1.2 times more than WT), the early (10 h) increased production could be achieved. Difficulty in finding good, hyperproducing, stable mutants might stem from the multistep pathway for BD formation when several genes are involved.

5.4 Purification of ALS

α-Acetolactate synthase (ALS-EC 4.1.3.18) enzyme is involved in 2,3-butanediol production and the biosynthesis of branched chain amino acids. Stormer (1975) reported two ALSs from *Enterobacter aerogenes*. One is valine sensitive and FAD-dependent, working at pH 8.0 and the other is pH 6.0, acetolactate-forming enzyme involved in 2,3-butanediol pathway. In the present study the α-ALS was purified to homogeneity, by subjecting the crude intracellular extract of *E. cloacae* to 50-90% ammonium sulfate fractionation, Sephadex G-200 gel filtration chromatography and the hydroxyapatite chromatography giving 5.0 fold purification with 7.8% yield. SDS-PAGE analysis showed the subunit molecular weight (≈60,000Da) where as native molecular weight, determined by gel filtration was ≈200,000Da. 50 to 90% ammonium sulphate saturation while purifying the α-ALS showed that the present enzyme protein was more hydrophobic in nature. In the literature the
native molecular weight of the protein was mentioned from 200-250kDa (Stormer, 1967; Holtzclaw and Chapman, 1975 and Eyong and Silverman, 1988). Keeping this in mind, the purification of α-ALS by Sephadex G-200 (gel filtration in a 70 cm long column) was used which helped in the separation of proteins according to their molecular weights. Hydroxyapatite chromatography enhance the fine resolution of the ALS proteins obtained by Sephadex purification eluted by 0.05 to 0.5M phosphate buffer gradient. Presumably negatively charged groups in the protein molecules bind to the Ca^{++} ions in the hydroxyapatite crystal lattice. Protein can be eluted from hydroxyapatite columns with phosphate buffer.

Stormer (1967) purified pH 6.0, acetolactate-forming enzyme from Aerobacter aerogenes. Total molecular weight of enzyme was 200,000 Da and subunit molecular weight approximately 58,000 Da.

Acetolactate synthase 1 purified from Escherichia coli K12, the ALS was composed of [(I lvB)3 (I lvN)3] gene products with a molecular weight of 200,000 and was made of 2 polypeptide chains, the larger one being ilv B gene product (M, 60,000) and the smaller being the ilv N gene product (Mr 11,000) (Eyong and Silverman, 1988).

Poulsen and Stougaard (1989) reported the purification of Saccharomyces cerevisiae ALS cloned in E.coli. There was a rather low overall recovery of ALS activity due to high lability of the enzyme. Native molecular mass was found to be 150 kDa and subunit molecular wt. was 75 kDa. So ALS appeared as dimer from yeast in E.coli after cloning.
α-ALS from *Lactococcus lactis* (Snoep et al. 1992) had native molecular weight 172 kDa. Subunit molecular weight was 62 kDa. He found that *Lactococcus* ALS enzyme was trimer of 62000 Da per subunit.

Yang & Kim (1993) purified the valine sensitive acetolactate synthase from *Serratia marcescens* ATCC 25419. The purified enzyme required TPP, Mg**+,** FAD, DTT (Dithiothritol) and glycerol for its full activity. *Serratia* ALS gave a single band on non-denaturing disc PAGE with a native molecular weight of 178000 while the subunit molecular weights were 62000 and 35000Da as determined by SDS-PAGE.

The catabolic α-ALS purified from *Leuconostoc mesenteroides* subsp. *cremoris* (Phalip, 1995) showed subunit molecular mass of 55,000 Da. Two peaks of ALS by gel filtration showed the molecular mass of 150 kDa (1st peak) whereas the 2nd peak corresponded to a mass of 50,000 indicating that the monomer was also active. This molecular mass, close to 55000-60000, is conserved for various ALS whether it is catabolic ALS from *Lactococcus lactis*, *Klebsiella pneumoniae* (Husby et al. 1971, Snoep et al. 1992) or a large subunit of anabolic ALS such as ALS I, II and III from *Escherichia coli* and *Salmonella typhimurium* (Friden et al., 1985, Squires et al., 1983, Wek et al. 1985).

The anabolic (pH 8 enzyme) and catabolic ALSs (pH 6 enzyme) are synthesized under different culture conditions (Halpern and Umbarger, 1959 and Gottschalk, 1979). The anabolic α-ALS is involved in valine, leucine and isoleucine synthesis. It catalyzes the formation of both α-acetolactate and
\(\alpha\)-aceto \(\alpha\)-hydroxybutyrate from pyruvate. Genes for the three isozymes of the anabolic \(\alpha\)-ALS encoded by \textit{ilv BM}, \textit{ilv GN} and \textit{ilv IH} from \textit{E.coli} have been isolated and sequenced. The \textit{ilv GMEDA} operon of \textit{Klebsiella aerogenes} has also been cloned but sequence of only the regulatory region is presently available (Harms \textit{et al.}, 1985). The anabolic \(\alpha\)-ALS enzymes of \textit{E.coli} consist of large subunits of 59, 60 and 62 kDa (encoded by \textit{ilv G}, \textit{ilv B} and \textit{ilv I} respectively) and small subunits of 11, 10 and 18 kDa (encoded by \textit{ilv M}, \textit{ilv N} and \textit{ilv H}, respectively). Blomqvist \textit{et al.} (1993) were the first to clone the genes encoding the catabolic (pH 6) \(\alpha\)-acetolactate-forming enzyme from \textit{Klebsiella terrigena}. The size of \textit{K.terrigena} \(\alpha\)-ALS was 60 kDa similar to the larger subunit of \(\alpha\)-ALS of \textit{A. aerogenes} described by Stormer (1975). Unlike the anabolic \(\alpha\)-ALS enzymes of \textit{E.coli}, the dimeric catabolic \(\alpha\)-ALS of \textit{A.aerogenes} and most likely that of \textit{K.terrigena} consist of only one type of subunit. \textit{K.terrigena} \(\alpha\)-ALS showed homology to large subunit of anabolic \(\alpha\)-ALS isozymes of \textit{E.coli}, especially in the N-terminal regions. Catabolic \(\alpha\)-ALS enzyme of \textit{K.terrigena} is able to convert pyruvate to \(\alpha\)-acetolactate when expressed in \textit{E.coli} but it was not involved in the formation of \(\alpha\)-aceto \(\alpha\)-hydroxy-buturate. It is tempting to speculate that the smaller subunit of anabolic \(\alpha\)-ALS isozyme has evolved especially for the formation of \(\alpha\)-aceto hydroxy-buturate. The third enzyme in the 2,3-butanediol pathway, AR (acetoin reductase) purified from \textit{Aerobacter aerogenes} consists of four equal size subunits of 25 kDa (Stormer, 1975). The last operon reading frame (gene \textit{bud C}) in the \textit{K.terrigena} operon codes for a protein of 25.6 kDa. AR catalyses
the reversible formation of 2,3-butanediol from acetoin and thus works as both reductase and a dehydrogenase. Cloning of genes involved in the bacterial butanediol pathway opens the possibility of elucidating the regulation of amino acid biosynthesis, pH and anaerobiosis. It also makes possible the use of genetic engineering to improve 2,3-butanediol production as well as other biotechnical processes of interest.

The effect of increasing pyruvate concentration on the E. cloacae ALS activity revealed that the acetoin synthesis does follow the simple Michaelis Menten type of saturation pattern and double reciprocal plot from saturation curve was linear in (Fig.30). The Hill plot (n=1.3) became >1.0 showing +ve co-operativity towards pyruvate as described by Koshland (1970). The Michaelis constants $V_{\text{max}}$ and $K_m$ were found to be 200 $\mu$M mg$^{-1}$ min$^{-1}$ and 20 mM in E. cloacae.

Benson et al. (1996) demonstrated the saturation kinetics of Ilv BN which showed that this enzyme has greater affinity for pyruvate ($K_m=8.3$ mM) than purified ALS of L. lactis subsp. lactis biovar diacetylactis ($K_m=50$ mM) (Snoep et al., 1992). A greater affinity for substrate should allow more generation of BD and acetoin from pyruvate, because greater substrate affinity may allow the diversion of pyruvate to the diacetyl, acetoin production pathway rather than acidic fermentation pathway. In wild type cultures, static growth allows increased activity of other enzymes (eg. Lactate dehydrogenase, pyruvate/formate lyase), thus reducing the pool of pyruvate available for $\alpha$-acetolactate synthase isozymes. ALS may not be able to act at this lower
concentration of pyruvate but *Iiv* BN is able to convert some of the pyruvate (Benson *et al.*, 1996).

The allosteric modulation of the enzyme permits avoidance of competition between pyruvate dehydrogenase complex and acetolactate synthase. The latter is constitutive and former is expressed in the sugar-limited cultures. Thus the ALS activity should be considered as removal of the excess of pyruvate when it occurred (Phalip *et al.*, 1995).

A substrate saturation curve for acetolactate formation as a function of pyruvate concentration was shown by Eyong and Silverman (1988) using ALS of *E.coli* K12. Double reciprocal plot showed the data are linear over a 40-fold range of pyruvate concentration. The $K_m$ value for pyruvate was 3mM, whereas this value was closer to 1.5mM, in case of (Schloss *et al.*, 1985) $V_{max}$ corresponds to about 40 μM of acetolactate min$^{-1}$ mg$^{-1}$ of protein.

On the other hand Schloss and Van Dyk (1988) showed that when two molecules of pyruvate condensed to form acetolactate, sigmoidal saturation kinetics were not observed. Presumably a consequence of the first pyruvate being added in a highly, as deduced from rather, small C-isotope effects for ALS II and the release of product (CO$_2$) before addition of the second pyruvate. In either case the apparent Michaelis-menton constant for saturation with pyruvate as a sole substrate is 11mM. This constant presumably reflects occupancy of the second pyruvate site, the first being silent. ALS II catalyses the homologus condensation of α-hydroxybutyrate and CO$_2$ with a Michaelis constant for α-ketobutyrate of about 10mM.
The Michaelis constant for pyruvate obtained with the purified enzyme was 12mM for homologus condensation of two pyruvate to form α-acetolactate and CO₂. The Km for pyruvate measured in crude extract was 7.6mM (Barak et al., 1988). The product formed by ALS III at equimolar pyruvate and α-ketobutyrate concentrations is approximately 50-fold greater towards α-aceto α-hydroxy butyrate versus α-acetolactate.

In our study the effects of inhibitory glycolytic pathway intermediates viz. 15mM ATP, 10mM glucose-6-phosphate, 10mM 3-phosphoglycerate and 10mM fructose-1, 6-bisphosphate (Fig.30) towards the substrate saturation curve of pyruvate showed the non-competitive inhibition as the Kᵦ value was not modified in the double reciprocal plot whereas the Vₘₙ value decreased by using these intermediates by 32.5, 58.4, 17 and 29% respectively.

α-ALS activity of E. cloacae was fully inhibited by sugars (0.1mM to 5mM conc.), xylose, starch, sucrose, lactose, rhamnose, maltose and succinic acid. EDTA and urea also inhibited the α-ALS activity. Hence the subunit of native enzyme was not found to work independently in E. cloacae ALS.

Cogan et al. (1981) and Phalip et al. (1995) reported the inhibitory effects of some intermediates of glucose catabolism (glycolysis) upon ALS activity. The physiological consequences of this behaviour seem to be limited since these products are transiently present in the cells. Furthermore, in these conditions when the concentration of the intermediates of glucose degradation is low the metabolism of lactic acid bacteria is switched towards paths other
than lactate formation such as C₄ compounds production (Thomas et al., 1979, Cogan et al., 1981).

Stormer (1988) showed that three molecules of co-carboxylase were found to be tightly bound to one molecule of the enzyme. The enzyme has a sharp pH optimum at 5.8. Apparent $K_m$ was only obtained in acetate and was estimated to be 5.9 – 6.6mM at pH 5.8. Buffers like phosphate, succinate, maleate and citrate cause sigmoidal kinetics and lower $V_{max}$. Phenyl pyruvate and glyoxylate are competitive inhibitors with $K_i$ values 0.11mM and 51 μM respectively. Competitive inhibition was also observed in the presence of ammonium sulfate but the curves were sigmoidal. When 2-oxobutyrate was added to the assay, a mixture of acetolactate and acetohydroxy butyrate were formed 2-oxobutyrate, acted as a competitive inhibitor with a $K_i$ of 5mM.

The purified ALS from Serratia marcescense ATCC 25419 exhibited sigmoidal saturation kinetics and non-linear double-reciprocal plot. The modified double-reciprocal plot yielded the apparent $K_m$ of 14.4 mM for pyruvate (Yang and Kim, 1993). Poulsen and Stougaard (1989) cloned the $als$ gene from Saccharomyces cerevisiae in E.coli. The presence of a peptide might also influence the kinetic behaviour of yeast ALS. The $K_m$ of approximately 4mM for pyruvate was found in permeablized yeast cells. In the latter study the authors (Magee and de Robinchon Swlmajster, 1968) were unable to observe any stimulation of yeast ALS activity by cofactors like FAD, thiamine diphosphate and Mg²⁺. But in contrast Poulsen and Stougaard (1989) observed hyperbolic saturation kinetics for all the cofactors. The Km value
reported for Mg2+ is the concentration of half maximal activation after gel filtration and extensive dialysis, which did not completely resolve ALS of that co-factor (the enzyme after dialysis showed approximately 20% of maximal activity in the absence of Mg++. The Kᵢ value was comparable with bacterial ALS (Grimminger & Umbarger 1979, and Schloss et al., 1985).

In the present study the Hill coefficient value was found to be n=1.3 showing positive co-operativity towards substrate binding of α-ALS from E.cloacae. The Hill’s co-efficient value of ALS from Serratia marcescence ATCC 25419 was n=2.0 suggesting the strong positive co-operativity in substrate binding (Yang and Kim, 1993). There have been occasional reports of negative co-operativity in the pyruvate saturation curve. Phalip et al. (1995) reported a Hill coefficient of 0.84 for catabolic ALS from Leuconostoc mesenteroides. Lower value of n=0.6 for recombinant ALS from Arabidopsis thaliana was also reported by Chang and Duggleby (1997). This shows that the ALS does not follow the Michaelis-Menten kinetics and negative co-operativity ascribed to interactions between the active sites of dimer.

Snoep et al. (1992) showed that Lactococcus ALS was allosteric with Hill coefficient of 2.4. Leuconostoc mesenteroides sub sp. cremoris enzyme is a trimer (3x55000) but is not allostric (n=0.84). Thus this structural difference could be linked to the fact that the Leuconostoc ALS remains active whereas the Lactococcus enzyme loses its activity when dissociated. The Leuconostoc enzyme in which subunits are probably readily dissociated and could work
independently. Strong tendency of dissociation of anabolic ALS has also been described in case of *E.coli* (Weinstock *et al.*, 1992).

Metal ion requirement has been reported for many acetolactate synthases (Malthe-Sorensen and Stormer, 1970; Snoep *et al.*, 1992 and Phalip *et al.*, 1995). Mg$$^{2+}$$ ions were found to be absolute requirement for ALS activity of *E.cloacae*, as the addition of EDTA (0.1 mM) completely inhibit the $\alpha$-ALS activity. Co$$^{2+}$$, Hg$$^{2+}$$, Ag$$^+$$ inhibited the ALS activity completely at 0.5mM. But on the other hand Tse and Schloss (1993) reported that Mn$$^{2+}$$ and Ni$$^{2+}$$ gave 133$\%$ and 50$\%$ activity as compared to Mg$$^{2+}$$, otherwise there is no great specificity in metal requirement and any of Mn$$^{2+}$$, Ca$$^{2+}$$, Cd$$^{2+}$$, Co$$^{2+}$$, Zn$$^{2+}$$, Cr$$^{2+}$$, Al$$^{3+}$$, Ba$$^{2+}$$ except Mg$$^{2+}$$ (0.1 to 10mM conc. requirement). Snoep *et al.* (1992) reported that ALS activity of *Lactococcus lactis* was completely inhibited by EDTA (5mM) but could be restored by adding Mg$$^{2+}$$ or Mn$$^{2+}$$ ions. *E.cloacae* ALS was inhibited by Hg$$^{2+}$$, Ag$$^+$$ indicate the breakage of s-s bonds which join the monomers (intersubunits) of intrasubunit. These dissociated subunits may be less or not active. The results correlate with the Hill coefficient ($n=1.3$) obtained in ALS of *E.cloacae* showing allostric ALS which was found to be inactive after dissociation.

TPP (Thiamine pyrophosphate) and Mg$$^{2+}$$ ions both were found as absolute requirement for ALS activity of *E.cloacae*, since the absence of TPP or addition of EDTA in the enzyme assay mixture showed no enzyme activity. It is believed that TPP is required by ALS from all species. It is generally used at concentrations 50$\mu$M or more without regard to whether a requirement has
actually been demonstrated (Pang and Duggleby, 2000). FAD, NADP and NADPH showed concentration dependent (1-10μM) inhibition of the *E. cloacae* ALS. Hence the ALS of *E. cloacae* was found to be FAD-independent. The results were similar to α-ALS of *Leuconostoc* (Stormer, 1975, Peng et al., 1992) but unlike the ALS from *Serratia marcescens* ATCC 25419 which was FAD-dependent (Yang and Kim, 1993; Phalip et al., 1995).

The reaction catalysed by ALS does not involve any oxidation or reduction so there is no obvious reason why the enzyme should require FAD and indeed, the activity of the catabolic ALS is independent of this cofactor (Stormer, 1968b). The role of FAD in the anabolic ALS has been speculated upon but there is, as yet, no clear-cut answer. Initially it was suggested that there might be a cyclic oxidation and reduction during the catalytic reaction (Stormer and Umbarger, 1964) but this possibility is now considered unlikely. There are two main hypotheses of why the anabolic ALS has retained its requirement for FAD. The first suggested function is that FAD is required for purely structural reasons; that is, unless FAD is present the active site cannot attain the correct geometry for substrate binding and/or catalysis to occur. The second hypothesis (Schloss and Aulabaugh, 1988) is that the FAD plays a protective role in the catalytic cycle. Under this proposal, it is necessary to prevent protonation of the α-carbanion during the step where the 2-ketoacid substrate binds. This is achieved by allowing the enamine to form a reversible adduct with FAD.
Structure of the enamine-FAD adduct of ALS (Schloss & Aulabaugh, 1988)

The difficulty with this hypothesis is that a similar adduct could not be formed by FADH₂ and yet replacing FAD with the reduced form of this cofactor actually leads to a 10% increase in ALS activity. Schloss and Aulabaugh (1988) have argued that this increased activity is due to elimination of the non-productive adduct formation but this could not protect the α-carbanion from protonation. Perhaps there are subtleties in the argument that could not be understood. Moreover, it provides no explanation of how the catabolic ALS is able to function without FAD. Thus, a definitive statement on the role of FAD is not yet possible.

In most studies, the FAD requirement is satisfied by adding this cofactor at a concentration of 2 to 100μM (Pang and Duggleby, 2000). But on the other hand where ALS of *E. cloacae* does not require FAD and their concentration dependent inhibition of ALS by FAD has not been described yet.
Strong positive co-operativity in the binding of pyruvate and affinity for it shows the regulation of BD and AMC production. Internal pyruvate is toxic to the organism in order to remove it α-ALS and other enzyme lactate dehydrogenase required and showed that NADH/NAD ratio and internal pyruvate play an important role in the catabolic fluxes of enzymes (Snoep et al., 1992). But in the present study NADP and NADPH did not help to improve ALS activity in E. cloacae inhibit the activity as the concentration increased (Snoep et al., 1992).

In some bacteria, pyruvate can be channelled via α-acetolactate into the formation of neutral compound 2,3-butanediol, the production of which is enhanced when the oxygen is limited and pH is lowered (≈5.8) (Johansen et al., 1975 and Magee & Kosaric, 1987). Three enzymes involved in 2,3-butanediol pathway are catabolic α-acetolactate synthase (ALS), acetolactate decarboxylase (α-ALDC) (EC 4.1.1.5) and acetoin (diacetyl) reductases (AR) also called butanediol dehydrogenase. In 1993, Blomoqvist et al. characterized the operon coding for α-ALDC, α-ALS and AR from Klebsiella terrigena and Enterobacter aerogenes. The genes located in operon transcribed in the order bud A, bud B and bud C, encoding α-ALDC, α-ALS and AR respectively. All the three genes have a potential ribosome-binding site 6 to 7 nucleotides in front of the translational start site. The size of operon was 3.4 kb, based on the nucleotide sequence. Optimal production of 2,3-butanediol was obtained under slightly acidic (pH 6), oxygen limited conditions and in the presence of acetate (Brown et al., 1972, Johansen et al.,
Pyruvate is channeled into 2,3-butanediol production when the acetate in the medium reaches a critical concentration and consequently 2,3-butanediol production prevents acetate overproduction and subsequent intracellular acidification.

Since the first enzyme synthesized from the operon is α-ALDC and not α-ALS, a rapid change in the metabolism of the cells towards 2,3-butanediol production would occur at the expense of valine leucine synthesis. In addition, reduction of acetoin to 2,3-butanediol serves to regenerate NAD$^+$ from NADH and because this reaction is reversible, AR also permits regeneration of NADH. Thus, 2,3 butanediol pathway serves to maintain the intracellular NAD$^+$/NADH balance in changing culture conditions. The bud A gene in both Klebsiella and E. aerogenes code for a protein of 29.2 kDa which corresponds to the previously reported size of the purified α-ALDC protein of Brevibacterium and Lactobacillus (Ohshiro et al., 1989 and Rasmussen et al., 1985).

Short-chain fatty acids, particularly sodium acetate have been found to be good inducer of α-ALS syntheses, in our laboratory and by other workers (Holtzclaw and Chapman, 1975), when it is added in the growth medium. Sodium acetate did not affect the ALS activity much i.e. 96.6% activity was retained at 0.1 mM concentration in case of E. cloacae while sodium formate, sodium propionate, sodium butyrate, sodium isobutyrate and sodium valerate inhibited the activity by 32, 56, 71, 32 and 100% respectively at this concentration. High concentration of sodium acetate (0.1-0.5%) inhibited the ALS activity. On the other hand Holtzclaw and Chapman (1975) found that
sodium acetate and sodium isobutyrate did not affect the activity of purified enzyme besides their help in the ALS synthesis.

Sodium acetate did not affect ALS activity at low concentration. But at high concentration it inhibits the activity of \textit{E.cloacae} ALS. Presumably sodium acetate might activate the genes like Als R responsible for the induction of ALS (Renna \textit{et al.}, 1993) with in the cell or may affect the ALDC rather than ALS activity or inhibit the enzymes involved in the mixed acid fermentation pathway.

In the present study the catabolic ALS (involved in 2,3-BD production) was feed back inhibited by leucine, which displayed more inhibitory effect (96\%) than isoleucine and valine 63\% and 25\% inhibition respectively at 0.1mM concentration. The activities of all ALSs except ALS-II of \textit{E.coli} and \textit{S.typhimurium} are inhibited at least by one branched chain amino acids. Valine was found to be a potent inhibitor at 4.4\mu M to 1.4 \mu M concentration (Magee and de Robinchon – Szulmajster, 1968).

Peng \textit{et al.} (1992) characterized the expression of a \textit{K. pneumoniae} gene, encoding an FAD-independent acetolactate synthase, which was not inhibited in the presence of 1mM valine. The pH optimum was 6.0 similar to acetolactate forming enzyme of \textit{E.aerogenes}. The enzyme activity decreased to 60\% when acetate buffer was substituted by the phosphate buffer of same pH and omission of Mn$^{++}$ and thiamine pyrophosphate did not seem to affect the activity of this pH 6 ALS. The Mn$^{++}$ requirement was further tested by including EDTA at a final concentration of 1mM. No effect was observed
Phalip, (1995) showed that catabolic $\alpha$-ALS from *Leuconostoc mesenteroids* did not require FAD/Mg$^{++}$, had pH optimum 5.3 and was involve in the 2,3-butanediol pathway but not in branched chain amino acid synthesis like $\alpha$-ALS of *Leuconostoc lacits* and *Klebsiella pneumoniae* (Cogan *et al.*, 1984; Peng *et al.*, 1992; Starrenburg and Hugenholtz, 1991). The $\alpha$-ALS was not inhibited by branched chain amino acids.

*Serratia* ALS was very sensitive to inhibition by valine (50% inhibition at 0.1mM). However the enzyme activity was also inhibited by isoleucine and leucine (50% at 1 mM) (Yang and Kim, 1993).

Stormer (1988) purified pH 6, acetolactate-forming enzyme from *Aerobacter aerogenes* which does not require FAD and was not inhibited by valine, leucine & isoleucine and was involved in the 2,3-butanediol pathway.

Benson *et al.* (1996) expressed plasmid - encoded *ilv BN* genes of $\alpha$-acetolactate synthase and ALS was found to be active in the presence of branched chain amino acids in the medium. They detected marked differences in acetoin and diacetyl production between static and aerated cultures. Previously it was studied that the production of these compounds increased 3 to 9 folds in the agitated cultures than static. This may be due to the increased activities of $\alpha$-acetolactate synthase and NADH oxidase in aerated conditions (Bassit *et al.*, 1993) or due to decreased pyruvate formate/lyase activity in these conditions (Starrenburg and Hugenholtz 1991). Snoep *et al.* (1992) showed that the purified enzyme was neither stimulated by the addition of FAD (1.5 $\mu$M) nor was subject to feedback control by branched chain amino acids.
acid like valine (1.5 mM), leucine (1.5mM) or isoleucine (1.5 mM) similar to anabolic α-ALSs (Eyong and Silverman, 1984; Durner and Boger, 1990 and Klemme and Schneider, 1990).

A drastic decrease in the α-ALS activity was observed with in 24 hours at 4°C in the enzymatic crude extract as compared to purified enzyme of E.cloacae. The storage of curde extract for 24 and 48 hr lead to loss of 70 and 90% activity respectively, while in case of the purified enzyme, 15 and 21% loss of activity was observed at 4°C and 25°C respectively in PBS (0.15M pH 7.0) after 24 and 48 hr while the 99% activity decreased after one month. Addition of TPP (80 µg/ml) and pyruvate 10mM stabilized the enzyme. After one week 71.68 and 53.72% α-ALS activity was retained at 4 and 25°C respectively. In regard to the finding of above results, TPP and pyruvate were added in each buffer 5 mg ml⁻¹ of BSA and 10-12% glycerol were also found to conserve the enzyme activity. Approximately 90% activity was conserved.

Stability of ALS from E.cloacae, in the current study, was protected by addition of TPP and pyruvate which maintained the enzyme in an active configuration. Bovine serum albumin enhanced the concentration of proteins and allow the preservation of the configuration although TPP and pyruvate are discarded. Increased amount of glycerol contents in the protein reduced the water contents and protect the protein from denaturation as well as make unfavourable environment for autocatalysis. Dilute enzyme looses its activity fastly as ion concentrations increased (Schloss et al., 1985). In a way, a steric
protective effect replaced a functional protective effect. Similar results obtained by ALS from *Leuconostoc mesenteroides* by Phalip et al. (1995).

Stormer (1967) experimented with crude and partially purified enzyme and showed that co-carboxylase, MgCl₂ and sodium pyruvate had a marked stabilizing effect on enzyme when the enzyme was heated to 60°C for 3 min., in the presence of all these compounds. Approximately 50% of the activity was intact after heating. He found that when the enzyme was stored with 50% glycerol at 0° for 18 months, there was no significant change in the activity. When acetolactate synthase from yeast was purified to apparent homogeneity, there was a rather low overall recovery of ALS activity, due to high lability of the enzyme. The individual column steps in the purification had a high recovery of ALS activity, but the enzyme lost its activity very fast and as the purity of the enzyme increased, it lost activity more rapidly (Poulsen and Stougaard, 1989).

This instability could be an explanation for the relatively low yield observed in the overall purification procedure, reported by Poulsen and Stougaard (1989). The individual column step in the purification had a high recovery of ALS activity, but the enzyme lost its activity very fast and the more pure enzyme became the faster it lost activity. After T-gel chromatography the enzyme lost up to 90% of its activity in about 8 hours. This resulted in 1% yield or less inspite of the high recoveries in individual step. There is no sign that the loss of activity was caused by proteolytic degradation of *E.coli* ALS because SDS-PAGE of an ALS active fraction and
rerun on SDS-PAGE of the same ALS (now inactive), fraction gave same bands indicating that there is some kind of denaturation (Poulsen and Stougaard, 1989).

Previous attempts to purify acetolactate synthase have been hampered by the extreme lability of ALS enzyme (Grimminger and Umbarger, 1979). This problem is particularly more with ALS isozymes (II & III) which binds FAD more avidly. The lability of isozyme II is complex and depends upon which of the tightly bound co-factor remains bound to the enzyme. In the presence of all three co-factors (FAD, thiamine pyrophosphate and Mg**), ALS II loses activity in an oxygen dependent process. Loss of enzymatic activity under these conditions does not seem to be due to the oxidation of enzymatic thiols as dithiothreitol did not protect the enzyme from inactivation and did not reverse the inactivation once it has occurred. Since the enzyme that has lost the activity under these conditions can be substantially reactivated (at least 10-fold) by extended dialysis against high concentrations of EDTA, it seems likely that inhibition could be due to the oxidation of enzyme bound thiamine pyrophosphate to the thiazolone (Schloss et al., 1985).

Thiamine–thiazolone–pyrophosphate, a potential reaction intermediate analogue, is an exceptionally potent, virtually irreversible inhibitor of a number of thiamine pyrophosphate-dependent enzymes (Gutowski & Lienhard, 1976). If the oxygen dependent loss of activity observed in the presence of thiamine pyrophosphate and metal is due to the formation of a tight-binding derivative of thiamine pyrophosphate, then the inhibition by
dialysis against a metal chelator is readily explained. In the absence of TPP and metal, the enzyme-FAD complex is quite stable. This form of the enzyme can survive several days incubation at room temp with no loss of activity. By contrast, enzyme stripped of FAD is unstable, especially at low protein concentration. The instability of this form of enzyme is different from that observed with its thiamine pyrophosphate – FAD – Mg** complex in that high salt prevents loss of activity. Stabilization of the FAD – free enzyme by the high salt is similar to the properties of ALS I (Grimminger & Umbarger, 1979). Although ALS II and ALS I exhibit similar activation constants for FAD (0.8 and 0.2 μM, respectively), the latter isozyme loses this cofactor far more readily. Losses of activity at low concentrations of FAD-free ALS II are likely to be due at least in part to surface adsorption. Intermediate dilution of the enzyme at low ionic strength, followed by dilution into an assay mixture, results in greater losses than dilution of the enzyme into an FAD-free assay mixture followed by initiating the assay with FAD.

The results of present work showed that ALS from *E.cloacae* was unusual as it has both catabolic and anabolic properties and might involved in the branched chain amino acid synthesis as well. ALS of *E.cloacae* was catabolic as it was involved in 2,3-BD production pathway, FAD-independent and worked at pH 6.0. SDS-PAGE showed that this enzyme form single subunit of 60 kDa which is present in the form of homotrimer (as the native molecular weight of the ALS protein was ~200 kDa). But this enzyme can also be anabolic because it is inhibited by branched chain amino acids (valine,
leucine and isoleucine) and worked well at pH 8.0. According to Gollop et al. (1989) suggested that anabolic enzyme should be termed as acetohydroxy acid synthase while the acetolactate synthase name should be conserved for catabolic enzyme. Since we have been working on the production of 2,3-butandiol for the last few years in our laboratory, present purified ALS of *E. cloacae* should be referred as catabolic acetolactate synthase as it is involved in 2,3-butanedial pathway.

5.5 Immobilization

Immobilization is simply a "tool" used by researchers to contain intact cells bound to an inert carrier within the fermentation vessel. Continuous immobilized cell fermentation system has the following advantages.

(i) Higher cell density per unit volume of the fermentation vessel gives higher fermentation rate.

(ii) The process may be operated without losing the entire population of cells.

(iii) Easy separation of immobilized cells from fermented broth, where the desired product can easily be collected, thus minimizing separation costs.

(iv) Higher cell densities combined with operation, decreasing the risk of reactor shut down due to contamination in case of free cells.

(v) Immobilized cells are less susceptible to inhibitory products, pH variations and nutrient depletion rather than free cells.

(vi) Smaller vessel volume which may lower the capital costs.
(vii) Systems with immobilized cells could be stepped up simply to meet the peak output requirement when needed. This would result in major cost serving over current technology utilizing free cells in large fermenters, which are adequate during peak seasons, but cannot be reutilised during off times.

(viii) A continuous process which may be performed beyond the nominal washout rate.

(ix) Improves the substrate utilization and gives constant product quality.

In the present study, *E. cloacae* cells, immobilized in alginate gel, were found to produce more (15%) BD than polyacrylamide-immobilized and free cells. The reason may be that alginate gel acts as an inert carrier support which generally protects the organism from excessive substrate, other inhibitors etc. rather than free cells. In polyacrylamide beads formation, probably, addition of TEMED and ammonium persulfate to the acrylamide solution mixed with microorganism suspension meets with exothermic reaction with the generation of heat which might reduce the viable cells in the respective gel. Moreover, TEMED may be toxic to the living cells and hence the production of BD was reduced in case of polyacrylamide gel.

Glucose was found to be the most suitable substrate for the production of 2,3-BD by free as well as immobilized cells rather than sucrose and molasses. This may be because of the fact that glucose is easily catabolized into pyruvate by glycolysis pathway and further pyruvate is converted into
acetolactate and then to acetoin and butanediol by enzymatic actions. Molasses might contain some inhibitors too.

Production of BD by immobilized cultures has had limited success to date (Magee and Kosaric, 1987). In our study, maximum (43.0 g) BD was produced per 100 g glucose utilized at the 24th h which was 87.6% of the theoretical value. Batch cultivation of *K. pneumoniae*, immobilized in k-carrageenan, resulted in the accumulation of 15 g L⁻¹ diol from 50 g L⁻¹ glucose at a productivity rate of 0.50 g L⁻¹ h⁻¹ (Chua et al., 1980). Qureshi and Cheryan (1989d) showed that *K. oxytoca* immobilized cells utilized 100 g L⁻¹ of glucose giving BD productivity of 0.40 g L⁻¹ h⁻¹.

The utilization of molasses for the production of 2,3-BD has also been investigated. Molasses are the residues left after crystallization of sucrose from sugar cane juices which can be stored in its concentrated form and can be utilized at optimum diluted sugar level prior to use. This substrate is being used widely for butanediol production but is generally considered to be uneconomical (Magee and Kosaric, 1987).

Aeration and agitation seem to play a key role in increasing the selective yield of 2,3-BD rather than AMC. At 0.5 VVM aeration rate and 24 h, BD production was found to be maximum i.e. 40 g/100 g glucose utilized. As the aeration rate increased from 0.5 to 2.0 VVM, AMC production gradually increased with decrease in BD production. Results were similar to the results obtained by Qureshi and Cheryan (1989). These authors described that inadequate aeration favours ethanol and lactate formation, while excessive
aeration results in high level of by products (especially acetate, acetoin etc.), poor usage of sugar and lowered BD production. Thus it is very important to optimise the level of aeration and agitation to maximize the yield and productivity of butanediol.

Various authors showed the effect of aeration on the production of BD as shown below:

**Effect of aeration rate on batch conversion of glucose**

<table>
<thead>
<tr>
<th>Glucose (g L(^{-1}))</th>
<th>Cells</th>
<th>Aeration</th>
<th>Temp. °C</th>
<th>pH</th>
<th>BD yield (g L(^{-1}))</th>
<th>AMC yield (g L(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Free</td>
<td>Anaerobic</td>
<td>37</td>
<td>-</td>
<td>3.5</td>
<td>NR</td>
<td>Yu &amp; Saddler (1982a)</td>
</tr>
<tr>
<td>40</td>
<td>Free</td>
<td>Finite air</td>
<td>30</td>
<td>6.5</td>
<td>8.2</td>
<td>NR</td>
<td>Yu &amp; Saddler (1982b)</td>
</tr>
<tr>
<td>107</td>
<td>Free</td>
<td>0.33 VVM</td>
<td>35</td>
<td>-</td>
<td>44.5</td>
<td>NR</td>
<td>Sablayrolles &amp; Goma (1984)</td>
</tr>
<tr>
<td>195</td>
<td>Free</td>
<td>0.33 VVM</td>
<td>35</td>
<td>-</td>
<td>88.0</td>
<td>NR</td>
<td>Sablayrolles &amp; Goma (1984)</td>
</tr>
<tr>
<td>40</td>
<td>Immobilized</td>
<td>0.5 VVM</td>
<td>30</td>
<td>5.8</td>
<td>17.6</td>
<td>0.8</td>
<td>This work</td>
</tr>
</tbody>
</table>

Sablayrolles and Goma (1984) used an 18-liter bioreactor sparged with 0.33 VVM air to examine the effect of \( K_La \) (oxygen absorption co-efficient) on the dissimilation of glucose by *K. pneumoniae*. At 100 h\(^{-1}\) \( K_La \), diol production was reported to be most efficient at the highest glucose concentration. At higher \( K_La \) conditions yield of biomass was markedly increased. Initial \( K_La \) was set at 30, 100, 150 and 300 h\(^{-1}\) where the diol formed was 0.44, 0.32, 0.20 and 0.17 g g\(^{-1}\) sugar respectively with a glucose concentration of 45 g L\(^{-1}\). So according to Sablayrolles and Goma (1984), the BD yield was based on initial glucose concentration and oxygen absorption co-efficient.
Vollbrecht (1982) defined a term "relative respiration rate" to measure the effect of aeration. It refers to ratio of the rate of oxygen uptake by the culture during the product formation to the maximum possible uptake rate. At the ratio 1.0, the oxygen demand of cells is fully satisfied. This ratio is thus index of effective aeration rate and is useful at very low oxygen demand. *Klebsiella pneumoniae* was grown between 0.05 and 0.20 VVM producing BD optimally at 0.10 VVM aeration rate. Acetoin was also maximally produced at the same rate. Vollbrecht (1982) also showed that higher aeration rate resulted in the formation of other organic acids like acetate, formate, succinic acid was found to be at 0.17, 0.17 and 0.20 aeration rates respectively.

Tsao (1978) examined the influence of DOT (%age of the medium's air saturation level) on product yields from 5% xylose. As the DOT was increased from 10% to between 20 and 30%, diol yield was reduced from 0.345 to 0.147 mol mol\(^{-1}\) of xylose consumed. Acetoin levels, however increased correspondingly from 0.062 to 0.256 mol mol\(^{-1}\), so that the net result was no real change in the combined yield of the two solvents (0.407 vs 0.403mol mol\(^{-1}\)).

The effect of aeration expressed as the oxygen supply rate \(K_{La}\) on the metabolism of xylose was reported by Jansen *et al* (1984a). By utilizing xylose (20 g L\(^{-1}\)) at pH 5.2 and 37°C, optimum diol i.e. 44% of theoretical value (productivity 0.44 g L\(^{-1}\) h\(^{-1}\)) was obtained at 0.014 mol L\(^{-1}\) h\(^{-1}\) oxygen supply rate. 53% diol yield was enhanced at 0.007 mol L\(^{-1}\) h\(^{-1}\) oxygen uptake.
Ace to in and BD are reported to be oxidation/reduction products of each other. The addition of reducing agents (cystein and ascorbic acid 0.1%) did not convert AMC to BD in this study but rather showed sharp decline in the overall yield of BD. This could probably be due to the effect of reducing agents on the enzymes involved in BD pathway or interference with the replenishment which holds a prime importance in the regulation of BD+AMC production (Maddox, 1988).

The catabolic α-ALS formed the acetolactate from pyruvate, acetolactate is decarboxylated by α-ALDC into acetoin which in turn is reduced into 2,3-BD by acetoin reductase. This latter step is accomplished by O₂ status of medium and end product distribution of BD and AMC (Groit et al., 1986). The stable flasks might be pre-requisite for the scaled up fermenter level. The oxygen status of the growing culture giving BD production can be changed by varying working volumes in the flask and agitation speeds. BD yield was found to be more when medium: flask volume ratio was 1:2.5. High and low ratios showed decreased amount of BD production (Delgado et al., 1989). Because at low working ratio oxygen level was increased thus increasing the cell mass but decreasing BD production. But at high working ratio lower oxygen solubility hindered with the proper cell growth resulting in decline in the BD production. Similarly, optimum BD yields were obtained at 180 rpm which could possibly be due to increase in the fermentation efficiency by continuously exposing new substrate to the culture and disseminating the
metabolic end products throughout the medium (Long and Patrick, 1963) besides elimination of the evolved CO₂.

Immobilized cells of *E.cloacae* gave 44.0 g BD per 100 g glucose utilized at the 24th h of growth and agitation rate of 180 rpm rather than stationary condition followed by agitation after different time intervals. In the latter case BD yield was reduced. On the contrary, Lee and Maddox (1986) showed that immobilized cells in static phase produced 50 g BD and agitation phase produced 30 g BD per 100 g sugar utilized.

Immobilized mutant cells produced 65.0 g BD per 100 g sugar utilized which was 1.5 times more than wild type immobilized *E.cloacae*. Semi-continuous batch fermentation showed that there was 0.8-20.0% decline in BD production by immobilized wild type cells and 7.0 to 30% decline in BD production by mutant of *E.cloacae* after the 7th cycle using various GYEP medium (described in section 4.5). But mutant cells gave 1.4 to 2.5 fold increased yield of BD as compared to wild type. In 1986, continuous reactor process was developed by Lee and Maddox for BD production by using cell of *K.pneumoniae* immobilized in alginate gel and they found that by this process no problems were encountered with gas removal or blockage resulting from excessive cell growth. The BD productivity reported in *K.pneumoniae* was 2.31 g L⁻¹ h⁻¹ (Lee and Maddox, 1986). On the other hand, Chua *et al* (1980) used an immobilized *E.aerogenes* in a continuous stirred tank reactor and reported productivity of 0.75 g L⁻¹ h⁻¹ from glucose substrate. Immobilized *B.polymyxa* gave BD productivity of 1.04 g L⁻¹ h⁻¹ from whey permeate (Shazes
and Speckman, 1984). In our study, immobilized wild type *E. cloacae* gave productivity of BD as 0.66 g\(^1\) L\(^{-1}\) h\(^{-1}\) and immobilized mutant of *E. cloacae* gave BD productivity as 1.08 g\(^1\) L\(^{-1}\) h\(^{-1}\) which was found to be maximum. Hence *E. cloacae* mutant can be used for further scaled up studies. While performing the semicontinuous experiments, after 4\(^{th}\) or 5\(^{th}\) cycle 50% of the alginate beads were broken down in GYEP I, II, III, IV, V and VII media except GYEP VI medium. This may be because the former media contained nutrient ingredients like yeast extract, soyameal, peptone, sodium pyruvate etc. beside glucose. These ingredients contained phosphate ions which in turn react with calcium alginate beads to form calcium phosphate, which lead to the breakage of beads. Moreover continuous agitation might also help in some mechanical breakage of the beads. But the latter medium, (GYEP VI), contained only glucose (main C-source) without all the ingredients and showed that very few beads were breaked down even after the 7\(^{th}\) cycle. Otherwise maximum number of beads remained intact. But the BD production in this medium was less as compared to other media.

Immobilized cells in our study, could be used atleast upto 7 cycle in a semi-continuous culturing process without significant fall in BD production and in this process, GYEP II medium for wild type while GYEP II & VII for mutant of *E. cloacae* were found to be best since these media are costeffective and maximum production was achieved even after 7\(^{th}\) cycle. GYEP VI medium for wild type immobilized *E. cloacae* was found to be cheaper with least decline (0.8%) in BD production after the 7\(^{th}\) cycle.
5.6 Future prospects

1. Process, on the production of 2,3-butanediol, can further be scaled up by continuous culturing of immobilized cells of *E. cloacae*.

2. In addition to α-ALS other enzymes viz α-ALDC and AR involved in 2,3-BD pathway can be characterized in order to find efficient inducers for the early synthesis of these all enzymes.

3. Multicopy number of genes, (viz *als* S, *als* D and *als* R) of α-ALS, α-ALDC and AR respectively, can be cloned in a suitable host to show the hyper expression of all these enzymes in the log phase of growth.

4. Commercial use of 2,3-butanediol can also be studied by:
   
   (i) Preparing the octane isomers of this compound and their use in fuels.

   (ii) Using the butanediol in cosmetics, fumigants preparation and as a carrier in drugs.

   (iii) Using it as an antifreeze (-60°C) agent.