Review of Literature
2. REVIEW OF LITERATURE

Studies on the production of 2,3-butanediol (2,3-BD) by fermentation, using micro-organisms, had started since 1906, when Harden and Walpole used *Aerobacter aerogenes* for the first time. Later, *Bacillus polymyxa* for 2,3-BD production was used (Morell *et al.*, 1945 and Blackwood *et al.*, 1949). Scientist's interest in the production of 2,3-butanediol again developed during world war-II due to the shortage of 1,3-butadiene, used in the production of buna type rubber (Long and Patrick, 1963). The developed processes could not be industrialized due to the competition from synthetic butadiene, obtained from petrochemical products (Maddox, 1988). This resulted in the discontinuation of biological production. But in 1970's, oil shocks and price rises again diverted the interest towards fermentation technology for 2,3-BD production.

2.1 2,3-Butanediol

2,3-BD is a compound which is present in three isomeric forms. These are D(−), L(+) and meso forms. D(−) and L(+) forms are the mirror images, common in physical properties except the direction of rotation of plane polarized light, while the racemic form has different physical properties than pure isomers (Ledingham and Neish, 1954).
Table 1: Physical properties of 2,3-butanediol (Ledingham and Neish, 1954)

<table>
<thead>
<tr>
<th>Constant</th>
<th>Meso 2,3-Butanediol</th>
<th>D(-) 2,3-Butanediol</th>
<th>Racemic 2,3-Butanediol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting Point (°C)</td>
<td>34.4</td>
<td>19.0</td>
<td>7.6</td>
</tr>
<tr>
<td>Boiling point 745 mm Hg (°C)</td>
<td>181-182</td>
<td>179-180</td>
<td>177.0</td>
</tr>
<tr>
<td>Specific rotation at 25°C (g/ml)</td>
<td>0.00</td>
<td>0.43</td>
<td>-</td>
</tr>
<tr>
<td>Surface tension at 25°C (dynes/cm)</td>
<td>-</td>
<td>30.61</td>
<td>-</td>
</tr>
<tr>
<td>Viscosity at 35°C (Centipoise)</td>
<td>65.6</td>
<td>21.80</td>
<td>-</td>
</tr>
<tr>
<td>Specific viscosity at 30°C</td>
<td>15.72</td>
<td>5.34</td>
<td>-</td>
</tr>
</tbody>
</table>

2.1.1 Sources of 2,3-butanediol

2,3-butanediol is found in many common materials, particularly foodstuffs. Foods and beverages, which are subject to the action of yeasts during their manufacture, contain 2,3-BD and acetoin. Various natural sources contain 2,3-BD in various concentration eg. beer (BD = 10.51 ppm), bread (10 ppm), butter (1.7 ppm), blood of higher animals (4 ppm), seedlings of higher plants (28 ppm), vinegar (500 ppm) and wines (1350 ppm) (Ledingham and Neish, 1954).

BD is a potential chemical having industrial importance with heating value of 27298 KJ/Kg, which is comparable to that of methanol (22081 KJ/Kg) and ethanol (29055 KJ/Kg), it is a highly valued fuel additive in addition to being a good solvent and extractant. Recent studies indicate that fermentation-derived 2,3-BD could also replace 1,4-BD in the production of
polyesters and polyurethane. Because of the low freezing point of the D-(-) isomer (-60°C), there is commercial interest in its use as an antifreeze agent (Magee and Kosaric, 1987; Syu, 2001). Generally organ cryopreservation is hindered by ice inflicted damage. Non freezing preservation of livers with 2,3-BD at subzero temperatures (-0°C) for longer period offered the advantage over conventional method (Scotte et al., 1996 and Soltys et al., 2001).

2,3-Butanediol used in the formation of polyurethane foams, octane isomers, synthetic fabrics such as perlon and tetralon. 1,3-Butadiene, which is produced by dehydration of BD could be polymerized to produce synthetic rubber (Tegtmier, 1989).

Table 2: Use of 2,3-butanediol (Tegtmier, 1989)

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Antifreeze agent, Extractant and solvent.</td>
</tr>
<tr>
<td>2. Dehydration</td>
<td>Butadiene and polymers formation.</td>
</tr>
<tr>
<td>3. Dehydrogenation</td>
<td>Methyl ethyl ketone, isomers of octane.</td>
</tr>
<tr>
<td>4. Esterification</td>
<td>Esters and polyurethane foams.</td>
</tr>
<tr>
<td>5. Dehydration</td>
<td>Diacetyl.</td>
</tr>
</tbody>
</table>

Dimerization of butadiene by the Diels-Alder reaction produces styrene, an important aromatic intermediate (Palsson et al., 1981). Styrene and butadiene are both important building blocks in polymer industry. Esters of butanediol and suitable monobasic acid could find use as effective plasticizers for thermoplastic polymers such as cellulose nitrate, cellulose triacetate, cellulose acetate butyrate, polyvinyl chloride, polyvinyl esters, polyacrylates and polymethyl acrylates (Maddox, 1988 and Syu, 2001).
Diaecetyl can be obtained by the oxidation of butanediol and forms an important flavour component in dairy products, especially butter and margarine (Rajagopalan et al., 1994; Romano and Suzzi, 1996 and Syu, 2001).

Further, butanediol has been found to be biodegradable representing its use in dyes besides an important environment friendly agent (Afschar et al., 1993).

2.1.2 Chemical properties

Butanediol can be converted to a variety of compounds chemically with diverse potential uses. These compounds are listed in the following table.

Table 3: Methods of preparation of various important compounds from 2,3-butanediol (Ledingham and Neish, 1954)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Method of preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetoin</td>
<td>Formed during fermentation when strongly aerated.</td>
</tr>
<tr>
<td>2,3-Butanediol diacetate</td>
<td>2,3-Butanediol &amp; sulfuric acid passed</td>
</tr>
<tr>
<td>2,3-Butanediol nitrate</td>
<td>D(-) 2,3-Butanediol esterified by nitric acid, dinitro sulfuric acid acting as catalyst.</td>
</tr>
<tr>
<td>1,3-Butadiene</td>
<td>2,3-Butanediol vapours passed over thorium dioxide at 350°C under reduced pressure</td>
</tr>
<tr>
<td>Diacetyl</td>
<td>Vapour phase oxidation or dehydrogenation of 2,3-butanediol over copper catalyst</td>
</tr>
<tr>
<td>Formal of 2,3-butanediol</td>
<td>Distills when acidified fermentation solution is heated with formaldehyde</td>
</tr>
<tr>
<td>Methyl ethyl ketone</td>
<td>2,3-Butanediol heated with sulfuric acid, the diol being added continuously as volatile product distils.</td>
</tr>
<tr>
<td>Methyl vinyl carbinol</td>
<td>2,3-Butanediol vapours passed over thorium dioxide under reduced pressure at 350°C</td>
</tr>
<tr>
<td>Methyl vinyl ketone</td>
<td>Vapours of acetoin, mixed with water and triethylamine, passed over alumina silica tungstic oxide catalyst</td>
</tr>
<tr>
<td>Polyester of 2,3-butanediol and pthalic acid</td>
<td>2,3-Butanediol heated with o-pthalic anhydride</td>
</tr>
</tbody>
</table>
2.2 Synthesis of 2,3-butanediol

2,3-butanediol can be synthesized chemically as well as biologically.

2.2.1 Chemical production of 2,3-butanediol

Chemically 2,3-BD is synthesized from D or (L) mannitol as described by Leon et al. (1952) and Rubin et al. (1952).

\[
\begin{align*}
D \text{ (or L) Mannitol} & \rightarrow 3,4\text{-isopropylidene-D (or L) mannitol} \\
& \rightarrow 2,3\text{-isopropylidene -D (or L) threitol} \\
& \rightarrow 1,4\text{-ditosyl 3-isopropylidene-D (or L) threitol} \\
& \rightarrow 1,4\text{-dideoxy 1,4-diido 2,3-isopropylidene-D (or L) threitol} \\
& \rightarrow 2,3\text{-isopropylidene -D (or L) butanediol} \\
& \rightarrow D \text{ (or L) 2,3-butanediol}
\end{align*}
\]

Butanediol can also be produced indirectly via acetoin using acetaldehyde (obtained from petrochemical sources) by acyloin condensation (Fieser and Fieser, 1961).

Chemical production suffers from major drawbacks including low conversion efficiency and rising petroleum costs. This factor, as well as, scarcity of non-renewable starting materials employed in chemical synthesis of 2,3-butanediol have led to the diversion of attention towards the fermentation route for the production of 2,3-butanediol.
2.2.2 Biological production of 2,3-butanediol by fermentation using micro-organisms

In early twentieth century Aerobacter aerogenes and Bacillus polymyxa were studied for the production of 2,3-butanediol (Harden and Walpole, 1906; Walpole, 1911; Harden and Norris, 1912). After this only occasional reports were seen till 1942. Interest developed again during the wartime due to shortages of 1,3-butadiene (Long and Patrick, 1963). However, the processes developed could not reach commercial level due to competition from synthetic butadiene obtained via petrochemical sources (Maddox, 1988). The oil shocks and price rise of 1970s again diverted the interest towards production of BD.

The two organisms that were initially implicated with industrial scale production processes included Aerobacter aerogenes and Bacillus sp. (Walpole, 1911; Gallagher and Stone, 1939; Stahly and Werkman, 1942; Katznelson, 1944; Perlman, 1944; Neish, 1945; Ward et al., 1945; Fratkin and Adams, 1946; Olson and Johnson, 1948; Blackwood et al., 1949; Sasaki, 1953; Ehlrich and Segel, 1959). Occasional studies were also performed using Klebsiella oxytoca, Aeromonas hydrophila and Aerobacillus polymyxa (Adams and Stanier, 1945; Blackwood et al., 1949), Brevibacterium saccharolyticum and Arthrobacter sp. (Buchanan and Gibbons, 1975; Ui et al., 1984a). Rarely, fungi have also been implicated with the production of 2,3-butanediol and the chief organisms reported include Rhizopus nigricans and Penicillium expansum (Fields and Richmond, 1967). A number of studies have been carried out in our laboratory relating to the production of closely related compound namely diacetyl, acetoin and 2,3-butanediol (Yadav and Gupta, 1975a; Yadav and
Gupta, 1975b; Yadav and Gupta, 1976a; Yadav and Gupta 1976b; Yadav and Gupta 1977; Gupta et al., 1978; Yadav et al., 1978; Gupta et al., 1979; Gupta et al., 1998). Saha and Bothast (1999) isolated Enterobacter cloacae NRRL B-23289 from local decaying wood/corn soil samples producing good yield of meso-2,3-BD (0.34 to 0.4 g/g available sugar) from multiple mixed sugar substrates. Important species of 2,3-butanediol producers were described in the table.

**Table 4: Important butanediol producers**

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eubacteriales</td>
<td>Eubacteriaceae</td>
<td>Enterobacter</td>
<td><em>E. aerogenes</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Aerobacter</em></td>
<td><em>E. cloacae</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Serratia</em></td>
<td><em>S. marcescens</em></td>
</tr>
<tr>
<td></td>
<td>Bacillaceae</td>
<td>Bacillus</td>
<td><em>B. polymyxa</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>B. licheniformis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>B. amyloliquefaciens</em></td>
</tr>
<tr>
<td>Pseudomonales</td>
<td>Pseudomonaceae</td>
<td>Aeromonas</td>
<td><em>A. hydrophila</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>A. indoles</em></td>
</tr>
</tbody>
</table>

**2.2.2.1 Biosynthetic pathway of 2,3-butanediol production**

Acetolactate synthase is the first common enzyme in the biosynthetic pathway leading to the 2,3-butanediol and branched chain amino acids viz. leucine, isoleucine and valine synthesis. Since the animals donot have mechanism to produce these amino acids, so they must take it from plants. It has long been known that certain species of bacteria can form acetoin (acetyl methyl carbinol) as a fermentation product of glucose. Silverman and Werkman (1941) described the mechanism of acetoin formation from pyruvate by action of enzymes including ALS, from crude extract of Aerobacter.
*aerogenes.* Juni et al. (1952a) resolved the crude acetoin forming enzyme system of *Aerobacter aerogenes,* into two distinct categories. The first is condensation of two molecules of pyruvate to form α-acetolactate and the second is decarboxylation of α-acetolactate to produce acetoin. Acetolactate is being used to produce acetoin and as precursor in valine biosynthesis. It can also be converted into diacetyl (chemically). Acetoin is converted to 2,3-BD by some bacteria e.g.; *Enterobacter, E. coli, Klebsiella* etc.

Main steps in the biosynthetic pathway of 2,3-butanediol are shown Fig. 1:

![Mixed acid-2,3-butanediol pathway](image)

**Fig. 1: Mixed acid-2,3-butanediol pathway.** Enzymes: 1, enzymes of glycolysis (and pentose phosphate pathway); 2, pyruvate kinase; 3, pyruvate-formate lyase; 4, acetaldehyde dehydrogenase; 5, ethanol dehydrogenase; 6, phospho-transacylase; 7, acetate kinase; 8, acetolactate synthase; 9, acetolactate decarboxylase; 10, acetoin reductase (butanediol dehydrogenase); 11, lactate dehydrogenase; 12, phosphoenolpyruvate carboxylase; 13, malate dehydrogenase; 14, fumarase; 15, succinate dehydrogenase; and 16, formate-hydrogen lyase.
Conversion of mono-saccharides to pyruvate introduces the routes involved in the generation of major products associated with the mixed acid – 2,3-butanediol fermentation.

2.2.2.1.1 Metabolism of pyruvate via the mixed acid-2,3-butanediol pathway

A common feature of the bacterial conversion of carbohydrates is the formation of multiple end products. Such is the case in the production of 2,3-butanediol. In addition to the diol, ethanol is surely present. A variety of organic acids may also be produced. The most common of these are acetate, lactate, formate and succinate. The metabolic routes by which these diverse compounds are produced from pyruvate are illustrated in Fig. 1.

2,3-BD is an organic compound having lots of industrial applications (Rehm, 1987). It can be used in the manufacturing of printing inks, perfumes, explosives, fumigants, moistening and softening agents besides carriers for pharmaceuticals (Magee and Kosaric, 1987). It can readily be dehydrated into methyl ethyl ketone (MEK) which is an industrial solvent and may find use as a liquid fuel additive and butadiene for manufacture of synthetic rubber (Long and Patrick, 1963). Butanediol can also be used as an octane booster in gasoline (Stinson, 1979). Several reductive elimination reactions allow the conversion of diol into alkenes (Corey and Winter, 1963; Josan and East wood, 1968). Alkenes (butenes) can be dehydrogenated to 1,3-butadiene in the presence of superheated steam as a diluent and a heating medium. Dehydration of 2,3-butanediol over a thoria catalyst produces butadiene,
although most of other dehydrogenation catalyst give methyl ethyl ketone as the main product (Winfield, 1945).

2,3-BD produced by microbial processes can exist in any of three stereoisomeric forms. The metabolic means by which these stereoisomers are formed has been the subject of much debate. Johansen et al. (1973) reported that a single enzyme isolated from *K. pneumoniae*, diacetyl (acetoin) reductase, conducted both the reversible reduction of acetoin to butanediol and the irreversible reduction of diacetyl to acetoin. This was in contrast to the work of Strecker and Harary (1954), in which a two enzyme system was proposed: a butanediol dehydrogenase (mediating the reversible reduction of acetoin to butanediol), and a diacetyl reductase (irreversible reduction of diacetyl to acetoin). The existence of two separate reductases was suggested by Ledingham and Neish (1954), one of which effected the reduction of D-(-) acetoin to mesodiol, while a distinct enzyme formed D-(-) diol from D-(-) acetoin.

\[
\text{D-(-) Acetoin} \rightarrow \text{L- (+) Acetoin}
\]

D-(-) Acetoin Reductase \[ \text{L- (+) Acetoin Reductase} \]

D-(-) -2,3-BD Meso-2,3-BD

Taylor and Juni (1960) proposed the existence of a three enzyme system with separate D-(-) and L- (+)-acetoin reductases. The reductases were said to be nonspecific with respect to acetoin stereoisomers, in that both would accept either D-(-) or L- (+) acetoin. The stereospecific nature of the resultant
butanediol would depend upon the particular reductases involved. Thus while D-(-) diol would be produced from D-(-) acetoin by the D-(-) acetoin reductase, this same enzyme would also produce mesodiol when L-(+) acetoin was used as its substrate. The corresponding activity of the L-(+) acetoin reductase is shown below.

![Diagram of acetoin metabolism]

The inability of extracts from *K. pneumoniae* and *S. marcescens* to oxidize D-(-) butanediol, and the ability of *B. polymyx* and *A. hydrophila* to do so (Hohnbentz and Radler, 1978), indicate that acetoin reductases are in fact stereospecific. It was concluded, therefore, that *K. pneumoniae* and *S. marcescens* possessed an L-(+) reductase, whereas *B. polymyx* and *A. hydrophila* utilize a D-(-) reductase.

A model for stereospecific reductases was thus proposed by Voloch *et al.* (1983) and is suitable for *K. pneumoniae*. However, extension to other species would necessitate the inclusion of a second D-(-) reductase capable of producing D-(-)-2,3-BD.
Metabolic diversity of the major diol producers enables a loose classification of butanediol fermentations based on the end product whose yield is second to that of the diol (Ledingham and Neish, 1954). Thus the diol-hydrogen fermentation, in which hydrogen and carbon dioxide are generated in large quantities, is typical of such species as \textit{B.polygonum}, \textit{K.pneumoniae} and \textit{A.hydrophila}.

\textbf{Table 5: Classes of butanediol fermentations}

<table>
<thead>
<tr>
<th>Fermentation class</th>
<th>Equation</th>
<th>Representative organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diol-hydrogen</td>
<td>Glucose = diol + 2CO$_2$ + H$_2$</td>
<td>\textit{B.polygonum}  \ \textit{K.pneumoniae}  \ \textit{A.hydrophila}</td>
</tr>
<tr>
<td>Diol-formate</td>
<td>Glucose = diol + CO$_2$ + formate</td>
<td>\textit{S.marcescens}</td>
</tr>
<tr>
<td>Diol-glycerol</td>
<td>2-Glucose = diol + 4CO$_2$ + 2glycerol</td>
<td>\textit{B.subtilis}</td>
</tr>
</tbody>
</table>

In \textit{S.marcescens}, little or no hydrogen is evolved, and the resultant accumulation of formic acid is the basis upon which this organism is categorized as a diol-formate producer.

Two important metabolic functions have been attributed to the formation of butanediol in microbial species. Regulation of intracellular pH
was proposed by Stormer (1968b). Prevention of acetate overproduction and subsequent acidification of the intracellular pH is mediated via the sensitivity of the acetolactate synthase to induction by dissociated acetate. Thus pyruvate is channeled into diol production when acetate reaches a critical concentration.

The optimal pH range for acetolactate synthase activity (5.5-6.5) corresponds with the pH range in which acetate is present in its dissociated form, suggesting that acetate performs an important role in the metabolic regulation of pyruvate catabolism. Kinetic evaluation of the purified acetolactate synthase from \textit{S.marcescens} has been done by Malthe-Sorensen and Stormer (1970) important role in diol formation. Reduction of acetoin to butanediol serves to regenerate NAD\(^+\) from NADH\(_2\) during the active catabolism of carbohydrates. When exhaustion of the substrate occurs, NADH\(_2\) is no longer formed, and the reversibility of the acetoin reductase reaction permits replenishment of NADH\(_2\) for cellular function. Butanediol thus serves as a reservoir of reducing equivalents.

\begin{itemize}
\item \textbf{Acetolactate decarboxylase, isolated from \textit{K.pneumoniae}, has a pH optimum in phosphate buffer of 6.2-6.4. (Loken and Stormer, 1970). At a pH of 5.8 (optimal for ALS) activity is about 75\% of the maximum. The stimulatory effect of Mn\(^{++}\) observed with crude extracts of the decarboxylase was lost upon purification of the preparation.}
\item \textbf{Under aerobic conditions, pyruvate is broken down to acetyl-CoA in enterobacteria by the action of pyruvate dehydrogenase multienzyme complex.}
\end{itemize}
This enzyme system is not synthesized in anaerobic environments, however, and is inhibited by the reduced form of the metabolic cofactor, NADH$_2$. Thus three main enzyme systems act upon pyruvate when the culture conditions are not fully aerobic. Lactate dehydrogenase, pyruvate-formate lyase and acetolactate synthase (pH 6 aceto lactate-forming enzyme). Pyruvate-formate lyase, induced by transition to anaerobic condition, is relatively unstable and is inactivated when the concentration of pyruvate becomes limiting. Rapid and irreversible inactivation of this system results from the introduction of oxygen to the culture. The formate generated by *K. pneumoniae* is further metabolized to CO$_2$ and H$_2$ by formate-hydrogen lyase complex. This complex is inhibited under aerobic conditions (Gottschalk, 1979).

Acetolactate synthase, acetolactate decarboxylase and acetoin reductase (butanediol dehydrogenase) effect the formation of 2,3-butanediol. Acetolactate synthase (pH6) enzyme conducts two step reaction. In the initial stage, pyruvate is complexed with thiamine pyrophosphate (TPP) to form acetyl-TPP. This compound subsequently is condensed with a second molecule of pyruvate to yield acetolactate.

The influence of acetic acid on dissimilation of glucose by *A. indologenes* described stimulation of BD production coupled with a decline in hydrogen evolution upon addition of the acid and using cell free extracts of *K. pneumoniae* (Happold and Spencer, 1952) similarly observed that acetic acid stimulate the production of acetoin. In a further development, Stormer (1968b), working with purified enzyme, showed that acetate caused induction
of all of the enzymes involved in the formation of butanedial from pyruvate. Maximum activity of all three enzymes was achieved at an acetate concentration of \( \approx 150\text{mM} \) (9.01 gL\(^{-1}\)). In each case activity was enhanced roughly 10-fold. Stormer (1977) also showed that dissociated form of acetate (CH\(_3\)COO\(^-\)) was responsible for induction of acetolactate synthase of \( K.pneumoniae \). Analogues of acetate substituted at the methyl group produced similar results. It therefore appears that the functional group responsible for the inducing effect is the COO\(^-\) of the ionized acetate. The esters of butanediol and suitable monobasic acid can be used as effective plasticizers for thermoplastic polymers such as cellulose nitrate, cellulose triacetate, cellulase acetate butyrate, polyvinyl chloride, polyvinyl esters, polyacrylates and polymethyl acrylates (Maddox, 1988 and Syu, 2001).

### 2.2.2.1.2 Theoretical yield of 2,3-butanediol

The theoretical yields of butanediol obtained from glucose can be calculated from the following equations:

\[
\text{Glucose} \rightarrow 2\text{CO}_2 + \text{NADH}_2 + 2\text{ATP} + \text{Butanediol}.
\]

Of the total carbon contained in the sugar, 2/3 goes to butanediol and 1/3 is lost as carbon dioxide. On a mass basis, the theoretical yield of butanediol from glucose is 0.5. The molar yield from hexoses is 1.0 (Magee and Kosaric, 1987).
2.3 Factors affecting acetolactate synthase and 2,3-butanediol production

2.3.1 Physical agents

2.3.1.1 Temperature

Temperature is an important factor affecting butanediol production, as the activity of various enzymes strongly affected by temperature (Esener et al., 1980; Esener et al., 1981; Bassit et al., 1995). From various studies it has been found that optimum temperature for BD production falls in the range of 30-35°C (Murphy et al., 1951; Maddox, 1988). Because BD production is considered to be a growth associated process (Esener et al., 1981), so optimum temperature for BD production is the same as that for maximum biomass yield. Nilegaonkar et al. (1992) found that optimum temperature for BD production from glucose using Bacillus
diol production by Bacillus polymyxa remained same at 25, 30 and 35°C respectively (Laube et al., 1984a). Barrett et al. (1983) examined the effect of temperature (30 to 37°C) on the production of 2,3-BD over 48 hour interval using Klebsiella pneumoniae, and obtained highest butanediol yields at 33°C, yields at 30 and 37°C being 95% and 33% as compared to the yield obtained at 33°C.

In case of Bacillus polymyxa, incubation temperature affected only the rate of biosynthesis of BD but not of acetoin, which was explained on the basis of significant difference between the temperature optima of the two enzymes involved (65-70°C and 37-40°C) respectively (Serenbrennikov, 1995).
2.3.1.2 pH

Optimum pH for butanediol production has been described in acidic range of 5.0-5.8 (Long and Patrick, 1963; Jansen et al., 1984a). While the alkaline conditions have been found to favour the production of organic acids thus resulting in a decreased butanediol yield (Neish and Ledingham, 1949). pH of the medium has been reported to exert effect via the influence on enzyme acetolactate synthase (Fond et al., 1985; de Mas et al., 1988 and Amanullah et al., 2001), increased pyruvate transport (McFall and Montville, 1989) and pH homeostasis via production of acetoin rather than acids (Tsau et al., 1992). Additionally, pH may effect the formation of enzymes which catalyse acetoin synthesis or may change cell permeability (Harvey and Collins, 1968; Branen and Keenan, 1972).

2.3.1.3 Aeration

Aeration is the important factor in butanediol fermentation. Aeration shows the characteristic of fermentation by controlling the three different pathways i.e. cell assimilation, respiration and fermentation (Harrison and Loveless, 1971; Ramachandran and Goma, 1987). Although butanediol is a product of anaerobic metabolism, aeration has been shown to enhance its production (Long and Patrick, 1963; Jansen et al., 1984b; Nilegaonkar et al., 1992). At higher oxygen transfer coefficient, cell growth is exponential and there is a decreased product formation. However, at lower dissolved oxygen concentration ~0, growth becomes linear and product formation has its greatest specific rate (Beronio et al., 1993; Silveira et al., 1993). Too much
aeration results in a higher growth rate and reduces the butanediol yield as the organisms start to obtain their energy via respiration rather than via fermentation and therefore, an increase fraction of carbon source is used for biomass production (Jansen et al., 1984; Alam et al., 1990).

Oxygen supply rate is also important because the ratio of oxygen demand to oxygen supply cannot the proportions of various metabolic products. Fermentation products excreted by *Klebsiella oxytoca*, other than butanediol include acetoin, ethanol, acetate etc. (Vollbrecht, 1982). In the absence of oxygen ethanol is produced approximately an equimolar amounts with butanediol. Presence of oxygen, however, inhibits ethanol production. Increasing oxygen supply rate towards the value of potential oxygen demand results in an increase in acetion: butanediol ratio (Fages et al., 1986). If the oxygen supply rate exceeds the microbial growth demand, oxygen is no longer a limiting factor and the only products of sugar metabolism are cell mass and carbondioxide (Pirt, 1958). Therefore, in order to maximize the butanediol production, a limiting but not zero supply of oxygen is required. Effect of aeration is of additional importance in diol fermentation, because it provides agitation also. Stirring increases the efficiency of fermentation by continuously exposing glucose substrates to culture and disseminating the metabolic end products throughout the medium i.e. at 0.33 VVM (volume of air/volume of medium/min) aeration rate 0.45 g g⁻¹ BD was produced (Sabalayrolles and Goma, 1984). Oxygen also acts as terminal electron acceptor in production of ATP via oxidative phosphorylation, ATP then
provide energy required in many of the reactions of cell synthesis, maintenance and product formation.

The effect of mixing and circulation time of O₂ at constant oxygen transfer rate (OTR=35 mmol h⁻¹) or K_L,a (volumetric transfer coefficient) is not applicable for scale up of *E. aerogenes* oxygen sensitive culture due to strong influence of reactor hydrodynamics under microaerobic conditions (Byun *et al.*, 1994). For a successful scale up of this microaerobic culture it is necessary to have an homogeneous oxygen supply over the entire reactor volume. Under the conditions of inhomogeneous oxygen supply and optimum liquid circulation time exists which gives a maximum production of 2,3-BD (Byun *et al.*, 1994).

2.3.2 Chemical agents

2.3.2.1 Substrates

Substrates used for butanediol production can be grouped into two categories – one comprising of pure sugars (monosaccharides, disaccharides and polysaccharides) and other of crude industrial wastes. While, earlier on, a good amount of work was carried out by using pure glucose as substrate (Table 6).
### Table 6: Butanediol production by various bacterial species using glucose substrate

<table>
<thead>
<tr>
<th>Organism</th>
<th>Glucose (g L(^{-1}))</th>
<th>Butanediol (g L(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. amyloliquefaciens</td>
<td>100.0</td>
<td>33.00</td>
<td>Barrett <em>et al.</em> (1983)</td>
</tr>
<tr>
<td>A. aerogenes</td>
<td>195.0</td>
<td>88.00</td>
<td>Sablayrolles and Goma (1984)</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>40.0</td>
<td>20.10</td>
<td>Yu <em>et al.</em> (1984b)</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>100.0</td>
<td>35.00</td>
<td>Ramachandran and Goma (1987)</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>107.0</td>
<td>44.50</td>
<td>Ramachandran and Goma (1987)</td>
</tr>
<tr>
<td>B. polymyxa</td>
<td>100.0</td>
<td>40.00</td>
<td>De Mas <em>et al.</em> (1988)</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>262.6</td>
<td>84.20</td>
<td>Qureshi and Cheryan (1989a)</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>94.3</td>
<td>38.10</td>
<td>Qureshi and Cheryan (1989b)</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>100.0</td>
<td>47.00</td>
<td>Nilegaonkar <em>et al.</em> (1992)</td>
</tr>
</tbody>
</table>

However, the need for efficient biological conversion of all available sugars present in biomass residues to fuel and chemical feedstocks, intended to compete economically with petrochemical products, has presently shifted the interest towards principle monosaccharides (Jansen *et al.*, 1984b; Schutz *et al.*, 1985; Lee and Maddox, 1986; Frazer and McCaskey, 1991) of cellulosic substrates (mannose, galactose besides glucose); hemicellulose (xylose, arabinose); whey (lactose); sugar crop (sucrose); starch; and Jerusalem artichoke (inulin and sucrose).

Industrial wastes like whey and molasses have been used successfully for butanediol production (Lee and Maddox, 1984; Schutz *et al.*, 1985; Afschar *et al.*, 1991). Other substrates like corn starch, acid hydrolysed starch, cereal mashes, barley corn and waste sulfite liquor (Wheat *et al.*, 1948; Martinez *et al.*, 1984 and Magee and Kosaric, 1987; Katznelson, 1994). Shortage of
non renewable resources generated the interest in the production of chemicals and fuels from lignocellulosic biomass which at present is available in large quantities on earth (Saddler et al., 1982 and Haspell, 1996).

In addition weed like water hyacinth which causes problem by impeding water flow and thus producing water logged areas could be used for feedstock for BD. This will change the status of weed from a nuisance to a potential provider (Taylor and Stewart, 1978; Wolverton and McDonald, 1979).

In our laboratory various agrowastes were used in the production of 2,3-BD by using Enterobacter cloacae and Klebsiella oxytoca (Amandeep, 1998) shown in Table 7.

**Table 7: Production of 2,3-BD+AMC from agrowastes**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>2,3-BD+AMC production per 100 g sugar utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. cloacae</em></td>
</tr>
<tr>
<td>Bagasse</td>
<td>39.7</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>34.9</td>
</tr>
<tr>
<td>Corn cobs</td>
<td>33.5</td>
</tr>
<tr>
<td>Water hyacinth</td>
<td>38.8</td>
</tr>
</tbody>
</table>

**2.3.2.2 Product concentration**

2,3-Butanediol is less toxic to producing organism (Fond et al., 1985). At higher concentration of BD, biomass formation is inhibited but product yield remains almost unaffected, which means that butanediol at high concentration strongly inhibits bacterial growth but has no effect on metabolic pathways leading to its production (Jansen et al., 1984a; Laube et al., 1984a, Sablayrolles and Goma, 1984). Butanediol concentration of 100 g/L has no
effect on its specific rate of production (Yu and Saddler, 1982). Micro-organisms have tolerance to high butanediol concentration in fermentation broth must showed that in the recovery procedures the purification of 2,3-BD become economic if butanediol is present at higher concentration in the fermentation broth.

2.3.2.3 Nitrogenous compounds

The requirement of nitrogenous compounds in the medium varies from one species to another. Long and Patrick (1963) found that Klebsiella pneumoniae and related species were less demanding in their nutritional requirements and gave satisfactory diol yields in media which contained sugar and inorganic salts only. Addition of 1% yeast extract and peptone in medium, increased the butanediol from zero (in only sugar containing medium) to 8.7 gL⁻¹ (Nilegaonkar et al., 1992). Yeast extract enhances diol yield but it is not economical to use in large quantities in commercial processes.

Urea in the medium have also been found to result in increase in diol yields. 1% urea to a defined medium resulted in 28% increase in butanediol yield, which was however less than that obtained using 1% yeast extract as supplement (Yu and Saddler, 1982a). Other nitrogenous sources which have been used as supplements in butanediol fermentation media were malt sprouts and brewer’s yeast (Kooi et al., 1948; Grover et al., 1990).

2.3.2.4 Acetic acid

Acetic acid has been found to effect all the three enzymes required for conversion of pyruvate to butanediol. Acetic acid is both a metabolic end
product of carbohydrate consumption and an important medium supplement which at low concentration has been found to enhance butanediol yield (Happold and Spencer, 1952; Bryn et al., 1973; Yu and Saddler 1982a; Barrett et al., 1983; Fond et al., 1985; de Mas et al., 1988, Zeng et al., 1990b). At higher acetic acid concentrations however, inhibition of growth has been reported (Yu and Saddler 1982b; Barrett et al., 1983; Zeng et al., 1990b). This inhibition was probably due to undissociated acetic acid rather than due to acetate ions and the effect has been found to diminish when medium pH is increased (Fond et al., 1985).

2.3.2.5 Lactic acid

Inhibitory effect of lactic acid on cell growth has also been reported by Ostling and Lindgren (1993), however extent of inhibition varies from one species to another (de Cardenas et al., 1985). Butanediol production appears to be concentration dependent which using lactic acid, lower levels <8 g L⁻¹ showed enhancement of butanediol production while higher concentration 15 g L⁻¹ resulted in almost complete inhibition of cell growth (Qureshi and Cheryan, 1989c).

2.3.2.6 Trace elements

Trace elements, Fe²⁺, Mn²⁺, Co²⁺ and Mg²⁺ have been found to stimulate butanediol production (Laube et al., 1984b; de Mas et al., 1988), while Ca²⁺, Fe³⁺ and Sn²⁺ have been found to exert an inhibitory effect (Laube et al., 1984b). Stimulatory effect of Mn²⁺ was explained on the basis of induction of acetolactate decarboxylase (Archihald and Fridovich, 1980).
2.3.2.7 Miscellaneous

Stimulation of butanediol production by the addition of acetaldehyde, pyruvic acid, \(\alpha\)-ketoglutaric acid, ethylene di-chlorotetra acetic acid, sodium azide and \(\alpha\)-chloromercuribenzoic acid has also been reported while cysteine, silver nitrate and acetaldehyde binding agents have been found to inhibit butanediol production (Chulkova and Kajanskaya, 1970; Collins and Speckman, 1975; Alkoyni et al., 1979).

2.3.3 Biological agents

2.3.3.1 Bacterial inoculum

Various workers have studied the effect of inoculum size on butanediol production. Yu and Saddler, (1982a) reported that although initial inoculum size of \textit{Klebsiella pneumoniae} increased the rate of fermentation of butanediol from xylose substrate, but yield of diol was found to be the same. Laube \textit{et al.} (1984a) however, found that increasing inoculum size from 2.5 to 5.0% resulted in no improvement in xylose substrate utilization or diol production by \textit{Bacillus polymyxa}.

2.4 Acetolactate synthase (ALS)

Enteric bacteria contain several acetolactate synthase (ALS, EC.4.1.3.18) isozymes also called acetohydroxy acid synthase (AHAS) and each of these isozymes seems to have diverged from a common ancestor (Friden \textit{et al.}, 1985; Wek \textit{et al.}, 1985) to function under different cellular conditions (McEwen \textit{et al.}, 1980; Dailey \textit{et al.}, 1986). The ALS catalyses the first pair of homologous reactions in 2,3-BD and valine/isoleucine biosynthetic pathways.
But it is not clear how or whether they regulate the two reactions they catalyse, which have the potential to interfere with each other. The conditions and their relation to enzyme structure and function remain important problems in microbial physiology.

ALS belongs to a family of homologous thiamine pyrophosphate (TPP)-dependent enzymes (Green, 1989), which catalyzes a process in which first common step is decarboxylation of pyruvate. The enzymes of this family differ widely with respect to the fate of TPP-bound “active aldehyde” intermediate formed by decarboxylation. This enzyme catalyses the condensation of hydroxy ethyl-TPP carbanion intermediate with a second α-keto acid to form two possible significant acetohydroxy acid products (Umbarger, 1987). The other enzymes with related activities like pyruvate decarboxylase allow protonation of the intermediate and their products are the respective aldehyde and pyruvate oxidases catalyse the oxidation of the hydroxy ethyl-TPP carbanion to acetyl-TPP, which enables it to participate in the decarboxylation of α-keto acids have been understood for many years (Brislow, 1958; Kluger, 1987). ALS form a primordial pyruvate oxidase explains the requirement of FAD in these non-oxidative enzymes (Chang and Cronan, 1988; Schloss et al., 1988). These enzymes also share common cofactors TPP, FAD and Mg^{++}.

ALS has been conserved across the species boundaries and substantial sequence similarities can be seen between enzymes of bacteria, yeasts and higher plants (Mazur et al., 1987). In enterobacteria, ALS occurs as a tetramer
of two large and two small subunits (Grimminger and Umbarger, 1979; Eoyang and Silverman, 1984; Schloss et al., 1985). Genes encoding for each of isozyme have been cloned and sequenced from *E.coli* (Lawther *et al.*, 1979; Squires *et al.*, 1981; Newman *et al.*, 1982; Squires *et al.*, 1983; Friden *et al.*, 1985; Wek *et al.*, 1985), from the yeast *Saccharomyces cerevisiae* (Falco and Dumas, 1985) and from higher plants *Arabidopsis* and *Nicotiana tabacum* (La Ross and Schloss, 1984). No small subunit has been demonstrated to be necessary for catalytic activity of either the yeast or plant ALS enzymes. Yeast has a single ALS isozyme which is encoded in the nucleus and ultimately is localized in mitochondria (Magee and Robinson, 1968; Ryan and Kohlnow, 1974). In higher plants, ALS is nuclear encoded and is localized in chloroplasts (Miflin, 1974; Chaleff, 1984 and Jones 1985). In each case the amino acid sequence contains an N-terminal region which is thought to serve as a transit peptide to direct the enzyme post-translationally into the subcellular compartment (Cleavage of transit peptide from the precursor protein yields the mature enzyme (Bascomb *et al.*, 1987). Three classes of herbicides-sulfonylurea (Chaleff and Mauvais, 1984; Ray 1984), imidazolinones (Shaner *et al.*, 1984) and the triazolopyrimidines (Hawkes *et al.*, 1984) target ALS enzyme. ALS enzymes from a wide range of organisms are sensitive to these compounds. Mutants resistant to these herbicides have been described in bacteria, yeast and higher plants (La Ross and Schloss, 1984; Haughns and Somerville, 1986) and resistance has been shown to be the consequence of an
altered ALS enzyme. *Arabidopsis thaliana* is commonly used as model system to study the gene expression in plant.

There are three isoforms of acetolactate synthase enzyme, ALS I, ALS II and ALS III of *Salmonella typhimurium* encoded by *ilv* BN, *ilv* GMEDA and *ilv* IH operons. Biochemical analysis has shown that all the three ALS isozymes are composed of two different polypeptides, a larger 50 kDa subunit and a smaller 10-17 kDa subunit (Squires *et al.*, 1983; Eoyang and Silverman, 1984; Lowther *et al.*, 1987). ALS I and ALS III are feedback-inhibited by valine (Guardiola *et al.*, 1977) ALS II is cryptic in the *E.coli* K-12 cells because of frame shift mutation in the *ilv* G-gene which encodes the large subunit of this enzyme (Lawther *et al.*, 1981). Genetic and biochemical evidences indicate that both the large and small subunits are catalytically essential (Lu and Umbarger, 1987; Squires *et al.*, 1981) in prokaryotes. The small subunit has also been shown to confer valine-sensitivity on the enzyme (De Felice *et al.*, 1974). In *Enterobacter* aerogenes two ALS isozymes have been reported one is valine sensitive and FAD dependent and the other is resistant and is thought to be the major enzyme for acetolactate synthesis in the 2,3-BD pathway (Stormer, 1975). *Salmonella typhimurium* expresses two isozymes, analogous to ALS I and ALS II (Squires *et al.*, 1983). Valine-sensitive ALS isozyme has been purified from *Serratia marcescens* with a molecular weight of 178000 (Yang & Kim, 1993). ALS belongs to two classes (i) the catabolic α-ALS catalyses the formation of acetolactate from pyruvate and (ii) the anabolic α-ALS which is involved in the biosynthesis of valine, leucine and isoleucine
and these enzymes are subject to feedback control by these amino acids (Basso et al., 1993 and Yang & Kim, 1993). The anabolic ALS requires FAD and MgCl₂ with optimum pH 8.0 for its activity whereas the catabolic ALS does not need these co-factors and works better at pH 6.0 (Juni, 1952; Malthe and Stormer, 1970). Lactococcus lactis and Leuconostoc have only anabolic ALS enzyme. While some organisms like Lactococcus lactis, Leuconostoc mesenteroides sub sp. cremoris 195 and Klebsiella pneumoniae possesses the sole catabolic α-ALS (Phalip, 1995). Enzymes like pyruvate decarboxylase (PDC) from Saccharomyces cervisiae showed the protein sequence similarities with α-ALS of Zymomonas mobilis and cytochrome-linked pyruvate oxidase from E.coli (ilv 1) and α-ALS (ilv 2 from S.cerevisiae) also have almost similar gene sequences. These all enzymes catalyse decarboxylation of pyruvate using TPP. General overall similarity suggests common ancestry for these enzymes (Green, 1989).

Some authors refer the ALS by different names. The catabolic enzyme has been referred to in the older literature as the “pH 6 acetolactate-forming enzyme” and more recently, as α-acetolactate synthase by Gollop et al. (1989) who suggested that the anabolic enzyme should be known as acetohydroxyacid synthase, while the name acetolactate synthase (ALS) should be reserved for the catabolic enzyme. The rationale for these suggested names is that the catabolic enzyme is capable of forming acetolactate only while the anabolic enzyme will form either of two acetohydroxyacids: acetolactate and acetohydroxy butyrate. Unfortunately, this nomenclature has not been widely
adopted and many publications continue to use the name acetolactate synthase for the anabolic enzyme (Duggleby and Pang, 2000).

The butanediol fermentation pathway is activated in bacteria by low external pH (5.5-6.5), low oxygen levels, the presence of an excess of acetate (Stormer, 1968a; Stormer, 1977; Johansen et al., 1975; Blomqvist et al., 1993; Mayer et al., 1995) and/or pyruvate (Tsau et al., 1992) and during the stationary phase (Renna et al., 1993). It has been argued that the pathway prevents intracellular acidification by diverting metabolism from acid production to the formation of the neutral compounds like acetoin and butanediol (Johansen et al., 1975; Tsau et al., 1992). The relative amounts of NAD\(^+\) and NADH within the cell may be regulated by the balance of acetoin and butanediol through the reversible reaction catalyzed by acetoin reductase. Hence, the significance of this pathway includes the maintenance of pH homeostasis, removal of excess pyruvate not used in biosynthesis, and regulating the NADH:NAD\(^+\) ratio within the cells. In addition, it has been shown that in *Lactococcus lactis* subsp. *lactis*, the activity of acetolactate decarboxylase is activated allosterically by leucine (Phalip et al., 1994), and its gene is located downstream of, and co-transcribed with, the branched-chain amino acid gene (*leu-ilv*) cluster (Chopin, 1993). The regulation of acetolactate decarboxylase activity and this genetic linkage suggest the importance of coordination between the butanediol fermentation pathway and branched-chain amino acid biosynthesis (Monnet et al., 1994; Goupil et al., 1996; Goupil-Feuillerat et al., 1997).
Despite the similarity of the reactions catalyzed by the catabolic and anabolic ALS, these enzymes can be distinguished easily. The catabolic ALS has been purified from its native source, and genes cloned and characterized. The purified catabolic enzyme is composed of a single subunit of about 60 kDa. It differs from the anabolic ALS in having a low pH optimum of about 6.0, is stimulated by acetate, does not require FAD, is not inhibited by the branched-chain amino acids and has no regulatory subunit (Stormer, 1968a; Stormer 1968b; Holtzclaw and Chapman, 1975; Snoep et al., 1992; Phalip et al. 1995). This differentiation is further supported by genetic characterization. The gene that encodes the catabolic ALS is found within the butanediol operon, no regulatory subunit gene is located downstream the gene, and the up-regulation of the operon corresponds to the conditions that activate the butanediol pathway (Blomqvist et al., 1993; Renna et al., 1993; Mayer et al., 1995).

2.4.1 Genetics

2.4.1.1 Bacteria

Among the bacteria, enzymes from enterobacteria are the most extensively studied both genetically and biochemically. At least three active ALS isozymes have been demonstrated in *E.coli* and *S.typhimurium*, namely ALS I, II and III encoded within the ilv BN (Wek et al., 1985), ilv GMEDA (Lawther et al., 1987) and ilv IH (Squires et al., 1983a) operons, respectively. In wild-type *E.coli* K-12 and *S.typhimurium* LT2, only two of these isozymes are expressed. The former does not have ALS II due to a frame-shift mutation.
(Lawther et al., 1981), and the latter is missing an active ALS III due to a mutation that creates a premature stop codon (Ricca et al., 1991) within the coding region of the catalytic subunit. Other cryptic genes have also been identified in *E.coli* (Jackson et al., 1981; Robinson and Jackson, 1982; Jackson et al., 1993). Due to the differences in their kinetic properties, substrate specificity, sensitivity to allosteric regulators, and hence the physiological functions of the various enterobacterial ALS isozymes, their expression is differently regulated (Umbarger, 1996).

Expression of the *ilv* BN operon is regulated by two mechanisms negative control via attenuation by the excess of valyl- and leucyl-tRNA, and positive control by cAMP and the cAMP-receptor protein (Sutton and Freundlich, 1980; Friden et al., 1982). Genes coding for the subunits of ALS II are located within the gene cluster *ilv* GMEDA. As mentioned earlier, ALS II is cryptic in *E.coli* K-12 due to a frameshift that leads to a premature stop codon in the middle of the catalytic subunit gene, *ilv* G. Expression can be restored by a frameshift mutation known as the *ilv* O mutation (Lawther et al., 1981). The translational stop codon of *ilv* G overlaps the regulatory subunit gene (*ilv* M) initiation codon in the four base sequence ATGA. Similar feature is also observed in the ALS subunit genes (*ilv* BN) of *Lactococcus lactis* subsp. *lactis*, which have a 9 bp overlap (Godon et al., 1992). Such overlaps have also been observed in genes specifying different polypeptides which are associated in multi-subunit enzyme complexes, presumably to ensure translational coupling leading to equimolar expression of the subunits (Das
and Yanofsky, 1984). The expression of ALS II is controlled by multivalent attenuation in which its expression is inhibited by the presence of all branched-chain amino acids (Harms et al., 1985). Lastly, the production of ALS III in E.coli is limited by excess leucine, mediated via the leucine-responsive regulatory protein (Wang and Calvo, 1993).

Mutations of wild type strains for the butanediol production or related compounds is comparatively less explored. Diacetyl-negative mutants were screened and characterized (Green et al., 1947; Burrow et al., 1968). Thiaminekinase-lacking mutants of Staphylococcus aureus showing no acetoin production (Sinha and Chatterjee, 1967) and B. subtilis mutants lacking acetoin reductase (Lopez et al., 1973) were produced. Comparison of three mutants of A. aerogenes with the wild type strain was used as a tool for investigating the physiological significance and biochemical route for the production of butanediol (Johansen et al, 1975). Mutants with increased expression of als SD operon were isolated, cloned and sequenced (Ranna et al, 1993).

2,3-Butanediol is produced in the stationary phase of bacterial growth because the gene encoding for the enzymes like α-ALS synthase are expressed during this phase (Johansen et al., 1975; Stormer, 1975; Tsau et al., 1992; Renna et al., 1993; Gupta et al., 1998), so efforts were made to prepone the production of 2,3-butanediol in order to shortened the fermentation time by manipulating the production of enzymes responsible for its formation. For
achieving this target workers have tried mutational methods as well as genetic engineering techniques.

Renna et al., (1993) cloned and sequenced the genes encoding the enzymes viz acetolactate synthase (alsS), acetolactate decarboxylase (alsD) and acetoin reductase (alsR) from Bacillus subtilis that regulates their expression. Als S and als D appear to lie on a single operon, while als R is transcribed divergently from als SD operon. Als R shows significant homology to the Lys R family of bacterial activator proteins and when als R is disrupted the als SD operon is not expressed. Transcriptional fusion of als S and als R revealed that Als R is required for the transcription of the als SD operons, which increases during stationary phase. Two mutants with change in the amino acid sequence in the Als R protein caused the increased expression of als SD operon in Bacillus subtilis.

The genes involved in the 2,3-BD pathway coding for α-ALS, α-ALDC and acetoin reductase have been isolated from Klebsiella terrigena and shown to be located in one operon and this operon has been shown to exist in Enterobacter aerogenes (Blomqvist et al., 1993). The bud A gene, coding for α-acetolactate decarboxylase, given in both organisms containing protein of 259 amino acids (87% similarity). The K. terrigena genes bud B and bud C, coding for α-ALS and acetoin reductase respectively were also sequenced. The 559 amino acids long α-ALS enzyme shows similarities to the large subunits of the anabolic α-ALS enzyme (Escherichia coli) encoded by the genes ilv B, ilv G and ilv I. The Klebsiella terrigena α-ALS also shown to complement an
anabolic α-ALS deficient *E.coli* strain for valine synthesis. The 243 amino acid long acetoin reductase has the consensus amino acid sequence for the insect-type alcohol dehydrogenase/ribitol dehydrogenase family and has extensive similarities with the N-terminal and external regions of three known dehydrogenases with one oxido reductases.

2.4.1.2 Fungi

A single *Saccharomyces cerevisiae* ALS gene, designated *ilv 2*, has been identified and cloned by complementation of an *ilv* yeast mutant (Polaina, 1984), and by its ability to confer low level resistance to the herbicidal inhibitor sulfometuron methyl in host cells when carried on a high copy number plasmid (Falco and Dumas, 1985). The *ilv 2* gene has been mapped to the right arm of chromosome XIII (*YMR108w*) (Petersen *et al.*, 1983). Other fungal ALS genes, which correspond to the catalytic subunit of the bacterial enzymes, have also been cloned (Jarai *et al.*, 1990). In contrast to *E.coli*, fungi have only one ALS isozyme, and no regulatory subunit gene has been found downstream of the cloned genes.

2.4.1.3 Plants

The identification of ALS as the site of action of sulfonylurea (Chaleff and Mauvais, 1984; Ray, 1984) and imidazolinone (Shaner *et al.*, 1984) herbicides greatly advanced the understanding of the enzyme and the biosynthetic pathway in which it functions in plants. The first two plant genes were isolated by Mazur *et al.* (1987) from *Arabidopsis thaliana* and *Nicotiana tabacum* using the yeast gene *ilv 2* as a heterologous hybridization probe.
Since then, a number of plant ALS genes have been cloned and characterized. These include those from *Brassica napus* (Rutledge *et al.*, 1991), *Zea mays* (Fang *et al.*, 1992) and *Gossypium hirsutum* (Grula *et al.*, 1995). These organisms vary in having a single ALS allele (*A. thaliana*), two copies of ALS (*N. tabacum* and *Z. mays*) to complex gene families (*B. napus* having five genes, and *G. hirsutum* having six).

### 2.4.1.4 Algae

ALS genes have also been cloned from algae, the more primitive representatives of the Kingdom Plantae. In contrast to those of higher plants, these genes are, in some cases, found to be located in the plastid genome (Reith and Munholland, 1993). With the availability of the complete nucleotide sequence of several algae plastid genomes, the genes for ALS regulatory subunits have also been identified (Ohta *et al.*, 1997).

### 2.4.1.5 Animals

A gene proposed to be the human homologue of the bacterial ALS catalytic subunit has been cloned (Joutel *et al.*, 1996). This gene was isolated accidentally in the process of mapping for the gene responsible for the condition known as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy).

This putative human ALS gene, which is interrupted by several introns, has been cloned from a cDNA library, and examined for ALS activity. The cloned gene failed to complement ALS-deficient *E. coli*. The protein, expressed in *E. coli*, exists exclusively in insoluble fraction and no ALS activity could be
detected (Duggleby et al., 2000). These results, together with the fact that animals are not believed to be capable of synthesizing the branched-chain amino acids, weaken the suggestion that this gene encodes a human ALS. However, the possibility that it catalyzes an ALS like reaction and functions in an as yet unknown pathway in animals cannot be excluded.

2.4.2 Amino acid sequences

2.4.2.1 Conserved residues

ALS genes have been identified and sequenced in a variety of plant, fungal, algal and bacterial species. In some, and perhaps all, species the enzyme is composed of a catalytic subunit and a smaller regulatory subunit. An alignment of the deduced amino acid sequences of a selection of 24 of these catalytic subunits showed that for any given pair, the calculated similarity score (Thompson et al., 1994) ranged from 99% (Bnal versus Bna3) to 17% (Ppu versus Mtu) with this latter pair showing 122 identities and 127 conservative substitutions. However, the overall alignment of all 24 sequences reveals only 27 identities. In part, the low number of absolutely conserved residues is due to a few sequences that differ substantially from the majority. It appears that anabolic and catabolic functions are performed by different forms of ALS that may be distinguished genetically and by their cofactor requirements. At least for the two Klebsiella proteins (Kpn and Kte), it is clear (Peng et al., 1992; Blomqvist et al., 1993) that they belong to the catabolic type. Although there are a number of sequence differences between the anabolic and catabolic types, no distinctive motif has been identified that can reliably place any given protein into one of the two types.
2.4.2.2 Transit peptide

The three plant (Ath, Nta and Bna) and two fungal (See and Spo) sequences are all substantially longer than the other proteins due to an N-terminal extension. Since nuclear genes encode the enzyme, it must be moved to the organelles after synthesis and it is probable that the N-terminal extension is involved in this intracellular trafficking. The unusual composition of these N-terminal regions, particularly the preponderance of serine residues, is typical of chloroplast and mitochondrial transit peptides (von Heinje et al., 1989).

The transit peptide targets the protein to the appropriate organelle and it is usually assumed that this transit peptide is cleaved during or after translocation. It is probable that the cleavage site is close to the region where homology with prokaryotic ALS sequences begins. Rutledge et al. (1991) have proposed that the cleavage involves removal of the first 70, 61 and 67 residues of Bna1, Bna2 and Bna3, respectively, so that each mature protein begins with the sequence TFXS[K/R][F/Y]AP that is common to all the plant ALS sequences shown in Fig.3.

2.4.3 Catalytic properties

2.4.3.1 Specificity and kinetic properties

After the initial decarboxylation step, ALS is capable of utilizing either pyruvate or 2-ketobutyrate as the second substrate. One of the important characteristics that distinguishes the isozymes of bacterial ALS is the specificity for the second substrate. The preference of the enzyme for either
pyruvate or 2-ketobutyrate in the second phase is defined by the specificity constant, $R$ (Barak et al., 1987).

$$R = \frac{(V_{AH8}/V_{AL})}{([2\text{-ketobutyrate}]/[\text{pyruvate}]).}$$

A wide range of substrate concentrations, pH, or the presence of inhibitor (valine or herbicides) do not affect this constant, which is an intrinsic property of the enzyme. Enzymes with a high $R$ value (>10) have a greater specificity for 2-ketobutyrate, while a value of less than 1 favors acetolactate synthesis.

The study of the substrate kinetics is described by Gollop et al. (1989) who examined the effect variation of the concentrations of pyruvate and 2-ketobutyrate using $E.coli$ ALS III. However, the catalytic cycle described shows, $\text{CO}_2$ release intervenes between the binding of the first and second pyruvate. Unless a very high $\text{CO}_2$ concentration is present, this step will be irreversible and the substrate saturation curve would be expected to be hyperbolic. This prediction agrees with the findings of most studies (e.g. $E.coli$ ALS II) and $K_m$ values in the range 1 to 20 mM are usually reported (Table 1).

Several papers have reported cooperativity kinetics for various forms of ALS. These include the enzyme from the barley (Miflin, 1971), $N.crassa$ (Kuwana et al., 1968), the bacterial anabolic ALS from $L.lactis$ (Snoep et al., 1992), $Serratia marcescens$ (Yang and Kim, 1993; see Fig.5), and $M.aeolicus$ (Xing and Whitman, 1994) and the catabolic enzyme from $A.aerogenes$ (Stormer, 1968a). The $L.lactis$ enzyme was later reported (Benson et al., 1996) to show hyperbolic kinetics but this difference may be due to different assay conditions.
2.4.3.2 Cofactor requirements

The enzymes that catalyze the decarboxylation of pyruvate and other 2-ketoacids, require thiamin diphosphate (TPP known as thiamin pyrophosphate) and a divalent metal ion as obligatory cofactors (Halpern and Umbarger, 1959). An unexpected observation, given that the reaction involves no oxidation or reduction, is that ALS also requires FAD (Stormer and Umbarger, 1964). It was later shown that some forms of ALS have no FAD requirement (Stormer, 1968b; Peng et al., 1992).

It is believed that TPP is required by ALS from all species although this has not been tested in many cases. More often than not, TPP is included in assays for the enzyme at concentrations of 50 μM or more without regard for whether a requirement has actually been demonstrated. However, in those cases where care has been taken to remove any TPP that might be bound to the enzyme, the activity can be reduced greatly or abolished entirely, and is fully restored upon adding back TPP. There is a hyperbolic dependence of activity upon concentration; from such experiments an activation constant or $K_m$ can be estimated (Table 3) and values ranging from less that 1 μM to greater than 200 μM have been reported.

The reactions catalyzed by almost all (Khaleeli et al., 1999) for an exception) TPP dependent enzymes can be written as involving cleavage of a carbon-carbon bond that is adjacent to a keto group. One of the products is
released while the other may be released, or remain bound to the enzyme where it undergoes further conversion. The ALS reaction is therefore typical; the bond broken is that linking the keto and carboxyl carbons of pyruvate and the product released is carbon dioxide (or bicarbonate). The second product is not released as acetaldehyde but is condensed with the 2-ketoacid substrate.

Fig. 2: Proposed catalytic cycle of ALS (Duggleby & Pang, 2000)
2.4.3.3 Metal ions

In common with all other TPP dependent enzymes, ALS requires a metal ion for activity. There is no great specificity in this requirement and any of Mn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Al$^{3+}$, Ba$^{2+}$ and Ni$^{2+}$ are active (Tse and Schloss, 1993) giving between 133% (Mn$^{2+}$) and 50% (Ni$^{2+}$) of the activity with Mg$^{2+}$. The metal ion requirement is usually satisfied with Mg$^{2+}$, generally at a concentration of 0.1 to 10 mM. However, it is not always easy to demonstrate that the metal ion is absolutely required due to the difficulty of removing all metal ions from both the enzyme and the assay solutions. In those cases where care has been taken to remove all sources of metal ions, the activity can be reduced to a small fraction of the initial value, but is completely restored upon adding back Mg$^{2+}$ (Schloss et al., 1985; Poulsen and Stougaard, 1989; Vyazmensky et al., 1996; Chang and Duggleby, 1998; Hill and Duggleby, 1998). There is a hyperbolic dependence of activity upon Mg$^{2+}$ concentration; from such experiments an activation constant can be determined. There are very large variations in the values that range from 3.8µM (E.coli isozyme II; Hill and Duggleby, 1998) through 280 µM (yeast; Poulsen and Stougaard, 1989) to 3.3 mM (E.coli isozyme III; Vyazmensky et al., 1996). It is not clear whether these variations represent true differences between ALS from various species or result from subtle differences in the assay conditions.
2.4.3.4 Flavin adenine dinucleotide (FAD)

The reaction catalyzed 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). Other enzymes that contain flavins with no redox role are rare but not unknown. One of these is glyoxylate carboligase (Chang et al., 1993) which catalyzes a reaction that parallels that of ALS. Rather than using two molecules of pyruvate, two molecules of glyoxylate are converted to tartronic semialdehyde and CO₂. This similarity, together with the extensive amino acid sequence homology to ALS, suggest that glyoxylate carboligase and ALS share a common evolutionary ancestor and that glyoxylate carboligase may be considered to be an ALS with unusual substrate specificity. Other examples of non-redox flavin independent enzymes include chorismate synthase (Macheroux et al., 1999) and hydroxynitrile lyase (Wajant and Effenberger, 1996) but in these cases, neither resembles ALS in structure nor in the reaction catalyzed. In an interesting parallel to ALS, there are two forms of hydroxynitrile lyase only one of which requires FAD (Wajant and Effenberger, 1996).

In most studies, the FAD requirement is satisfied by adding this cofactor at a concentration of 2 to 100 μM, although it is not uncommon for it to be omitted entirely. Presumably, this is not because the enzyme does not require FAD; rather it is bound sufficiently tightly that it is not lost from the enzyme during extraction and purification. Indeed, removal of FAD may
require special action such as treatment with activated charcoal (e.g. Hill and Duggleby, 1998). Once this is done, the enzyme shows little or no activity but can be made fully active by adding back FAD. The hyperbolic reactivation curve yields an activation constant and the values that have been determined for various forms of ALS. In all cases these are in the high nM to low µM range and these high affinities are consistent with the fact that the enzyme is frequently found to be active in the absence of added FAD.

2.4.3.5 Feedback regulation

ALS is the key control point within the branched-chain amino acid biosynthetic pathway. Because of the critical role to ensure a balanced supply of the amino acids, ALS expression and enzymatic activity within cells are tightly controlled by various mechanisms. One of the mechanisms involves the control of the enzyme at the transcriptional level, which has been dealt with in Section 3. The other mechanism, as in many biosynthetic pathways, regulates ALS activity by end-product feedback inhibition. The activities of all ALS, except ALS II of *E.coli* and *S.typhimurium*, are inhibited by at least one of the branched-chain amino acids. The inhibition is described as non-competitive in relation to pyruvate (Magee and de Robichon-Szulmajster, 1968b; Glatzer et al., 1972; Takenaka and Kuwana, 1972; Proteau and Silver, 1991). In all the cases examined in bacteria (Barak et al., 1988; Proteau and Silver, 1991; Yang and Kim, 1993), fungi (Magee and de Robichon-Szulmajster, 1968b; Glatzer et al., 1972; Takenaka and Kuwana, 1972; Pang and Duggleby, 1999), and algae (Oda et al., 1982; Landstein et al., 1993), valine is clearly the most
potent inhibitor amongst the branched-chain amino acids. The reported apparent $K_i$ for valine ranges from 4.4 $\mu$M to 1.4 mM. In contrast, ALS from higher plants is regulated slightly differently from the enzymes in lower organisms. In plants, leucine is an equally good, and sometimes better, inhibitor than valine (Miflin, 1971; Durner and Boger, 1988; Singh et al., 1988; Southan and Copeland, 1996). In addition, the inhibitory effect of the branched-chain amino acids used in combination is greater than the additive effect of them added singly (Miflin, 1971; Durner and Boger, 1988). Such synergism has not been reported for ALS for bacteria, yeast or algae.

2.4.4 Subunits

The subunit composition and structure of $\alpha$-acetolactate synthase from various sources appears to different.

2.4.4.1 Bacteria

The functional genes of ALS isozymes of $E. coli$ and $S. typhimurium$ have been cloned and characterized, and each consists of two structural genes arranged within a operon (Squires et al., 1983a; Squires et al., 1983b; Wek et al., 1985; Lawther et al., 1987). Each set of genes encodes a large subunit of about 60 kDa and a smaller (about 10-17 kDa) subunit, with both subunits essential for full ALS activity. ALS purified from $S. marcescens$, is also composed of two types of subunit although in this case the smaller subunit (35kDa) is considerably larger than that from the enteric bacteria (Yang and Kim, 1995).
The physical association of the large and small subunits was demonstrated initially for *E. coli* ALS I by the co-precipitation of both subunits from a crude cell extract using an antibody against the large subunit (Eoyang and Silverman, 1984). The three active isozymes have been purified to near homogeneity (Schloss *et al.*, 1985; Barak *et al.*, 1988; Eoyang and Silverman, 1988; Hill *et al.*, 1997). Both subunits are consistently purified together with the ALS activity. Furthermore, studies including quantitative analysis of SDS-PAGE (Eoyang and Silverman, 1984), carboxymethylation and gel filtration (Schloss *et al.*, 1985), association kinetics and radioactive labeling (Sella *et al.*, 1993) have revealed that the two polypeptides are in 1:1 stoichiometry. The native size of the isozymes is between 140-150 kDa leading to the conclusion that the enzyme has an $\alpha_2\beta_2$ quaternary structure consisting of two small subunits.

Studies on the properties of the subunits have been carried out by alteration of the subunit genes by mutation (De Felice *et al.*, 1974; Eoyang and Silverman, 1986; Lu and Umbarger, 1987; Ricca *et al.*, 1988; Vyazmensky *et al.*, 1996), expression of individual subunits and their reconstitution (Weinstock *et al.*, 1992; Sella *et al.*, 1993; Vyazmensky *et al.*, 1996; Hill *et al.*, 1997), or by chemical inactivation (Silverman and Eoyang, 1987). The mutation studies were done by the complementation of ALS-deficient strains of bacteria and activity measurements in bacterial crude extract, while the remaining studies have been carried out using either crude extracts or purified subunits.
The pure ALS II catalytic subunit exists predominantly as dimers (Hill et al., 1997). In contrast, the catalytic subunits of ALS III were observed to migrate as monomers in gel filtration, but the authors express some surprise at this result given that these subunits are catalytically active (Vyazmensky et al., 1996).

As ALS small subunit has been implicated in conferring valine sensitivity on the enzyme (De Felice et al., 1974; Eoyang and Silverman, 1986; Weinstock et al., 1992; Sella et al., 1993) and therefore refer to it as the regulatory subunit. The residual activities of the catalytic subunit of ALS I and III are completely valine insensitive. On reconstitution with the regulatory subunits, valine sensitivity is restored. The regulatory role of the small subunit was further confirmed by Vyazmensky et al. (1996) with highly purified subunits of ALS III.

It is known that the enterobacterial ALS II isozyme is insensitive to valine feedback regulation but curiously it has an absolute requirement for its regulatory subunit. The specific activity of the catalytic subunit is massively enhanced upon reconstitution with the regulatory subunits (Hill et al., 1997). This association apparently stabilizes the active conformation of the catalytic subunit leading to an increase in the turnover number. The interaction is highly specific in that reconstitution with non-matching subunits between isozymes does not occur (Weinstock et al., 1992). The reconstitution of the catalytic subunit with its regulatory subunit follows simple saturation kinetics in ALS III (Vyazmensky et al., 1996), but is positively cooperative in ALS II.
In both cases an excess of regulatory subunits is required to fully reconstitute the catalytic subunits. In summary, the regulatory subunits of bacterial ALS control ALS activity by conferring upon it end-product feedback inhibition, and by increasing substantially the enzymatic activity.

On apparent exception to this rule is ALS from *Methanococcus aeolicus*. The purified enzyme contains no detectable regulatory subunit although the enzyme is sensitive to inhibition by valine and by isoleucine (Xing and Whitman, 1994). However, later it was shown that a genetic arrangement similar to that in other bacteria with a probable regulatory subunit open reading frame just downstream of the catalytic subunit gene. Thus, it seems likely that the regulatory subunit was present, but not detected, in the purified enzyme although this proposition has yet to be verified experimentally (Duggleby and Pang, 2000).

2.4.4.2 Fungi

In contrast to the bacterial enzymes, the structure and biochemical properties of ALS from eukaryotes are not well characterized, and in most cases the enzyme has been studied in crude extracts only. A few fungal ALS genes have been cloned (Polaina, 1984; Falco *et al*., 1985; Jarai *et al*., 1990) by complementation and the deduced amino acid sequences (except for the N-terminal transit peptide) are collinear with those of bacterial ALS catalytic subunits.

2.4.4.3 Plants

As with fungi, the plant ALS genes that have been cloned over the past decade correspond to the catalytic subunits of the bacterial enzyme. These
genes encode a polypeptide with a molecular mass of about 72 kDa, which is about 10 kDa larger than the bacterial catalytic subunit. As expected, the extra 10 kDa is contributed by an N-terminal organelle-targeting sequence. This transit sequence has been shown in vivo to be cleaved upon translocation into the chloroplast and in vitro by Western blotting with anti-ALS antisera (Singh et al., 1991). In the latter experiments, the antisera cross-react with a 65 kDa protein found in a wide variety of monocotyledonous and dicotyledonous plants. In contrast, SDS-PAGE analysis of the purified ALS from barley (Durner and Boger, 1988) and wheat (Southan and Copeland, 1996) revealed that the large subunits have molecular masses of 57 kDa. The reason for the apparently smaller molecular mass of the large subunit of the purified enzyme is unknown.

2.4.5 Purification

In order to conduct thorough and accurate enzyme characterization, a large amount of relatively pure ALS is required. The enzyme can be obtained either from its native source or by over-production in a heterologous expression system. Purification from its native source will be difficult if the enzyme constitutes only a very small proportion of the total protein. More success has been achieved by over-expression in E.coli. However, this strategy can only be applied if the structural gene(s) of the ALS have been cloned. A second potential drawback is that the prokaryotic host cells might not provide the correct machinery for any post-translational modification and processing that might occur in the native cells.
2.4.5.1 Natural sources

ALS from bacteria, and particularly the \textit{E.coli} isozymes, are purified more easily than eukaryotic ALS. In \textit{E.coli}, due to the presence of multiple isozymes, mutant strains that have only one active ALS are usually used as the starting material. Grimminger and Umbarger (1979) reported the purification of ALS I from such a mutant. A relatively low yield of 8\% was achieved after five purification steps, even though a fairly high specific activity (34 U/mg) was obtained. Such mutants are not required if the bacterial species have only one ALS (Xing and Whitman, 1994), or the multiple ALS activities can be separated easily during purification (Yang and Kim, 1993). Yields as high as 27\% or as low as 1\% (Xing and Whitman, 1994) have been reported, depending on the complexity of purification and lability of the enzyme. The table 8 showed the purification of $\alpha$-ALS from various microorganisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Yield (%)</th>
<th>Purification fold</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Aerobacter aerogenes}</td>
<td>1,526</td>
<td>31800.0</td>
<td>27.0</td>
<td>120.0</td>
<td>Stormer (1967)</td>
</tr>
<tr>
<td>\textit{Bacillus subtilis}</td>
<td>64,127</td>
<td>8280.9</td>
<td>35.1</td>
<td>12.6</td>
<td>Holtzclaw &amp; Chapman (1975)</td>
</tr>
<tr>
<td>\textit{Escherichia coli} K-12</td>
<td>751</td>
<td>30.0</td>
<td>20.0</td>
<td>23.0</td>
<td>Eoyang &amp; Silverman (1988)</td>
</tr>
<tr>
<td>\textit{Saccharomyces cerevisiae} ALS from recombinant \textit{Escherichia coli}</td>
<td>2.3</td>
<td>0.17</td>
<td>1</td>
<td>1.8</td>
<td>Poulsen &amp; Stougaard (1989)</td>
</tr>
<tr>
<td>\textit{Serratia} marcescens ATCC 25419</td>
<td>-</td>
<td>-</td>
<td>23%</td>
<td>222.5</td>
<td>Yang &amp; Kim (1993)</td>
</tr>
<tr>
<td>\textit{Leuconostoc mesenteroides}</td>
<td>0.84</td>
<td>0.844</td>
<td>5.4</td>
<td>22.2</td>
<td>Phalip \textit{et al.} (1995)</td>
</tr>
</tbody>
</table>

Table 8: Purification of acetolactate synthase from various organisms
The pH 6 acetolactate-forming enzyme has been isolated from *Aerobacter aerogenes* and crystallized in the presence of sodium pyruvate, co-carboxylase and MgCl₂ (Stormer, 1967). Two different crystal forms, one plate-shaped and another needle-shaped, are described. The latter crystalline form, which is developed in the absence of (NH₄)₂SO₄, is slightly soluble at neutral pH and has low specific activity when brought into solution at high or low pH. The needle shaped crystals are purified enzyme protein and not an artifact, has been described by Stormer (1975). The degradative α-ALS enzyme was partially purified from *Bacillus subtilis* (Holtzclaw and Chapman, 1975). This enzyme when assayed in phosphate buffer (pH 7.0), the activity stimulated by acetate buffer and inhibited by sulfate. When assayed in acetate buffer (pH 5.8), activity was inhibited both by sulfate and phosphate. ammonium sulfate fractionation, gel filtration and hydroxyapatite chromatographical techniques have been applied for the purification of α-ALS from various organisms eg *Aerobacter aerogenes, Bacillus subtilis, E.coli, Serratia marcescense* and *Leuconostoc mesenteroides* (Stormer, 1967; Stormer, 1975; Holtzclaw and Chapman, 1975; Eoyang and Silverman, 1988; Yang and Kim, 1993 and Phalip et al., 1995). Homogeneity and purity of the α-ALS has been determined by SDS-PAGE and silver staining procedures (Eoyang and Silverman, 1988; Yang & Kim, 1993 and Phalip et al., 1995). To simplify the purification and to improve the yields, the level of ALS can be raised by the introduction of ALS genes in high copy number plasmids (Barak et al., 1988; Eoyang and Silverman, 1988) or under the control of a strong promoter (Hill et al., 1997).
N-terminal amino acid analysis of large as well small subunits (incase of anabolic α-ALS) has been performed on a gas phase sequencer (Falco et al., 1985 and Yang & Kim, 1993). The amino terminal sequence is basic coding sequence of polypeptide contains 2061 base pairs. There are no acidic residues among the first fifty-five amino acids, from the first ATG, but there are seven organizers, three lysines and one histidine. From the second ATG no acidic residues appear among the first twenty nine amino acids, which include four arginine and one lysine. Starting from either ATG there is a stretch of four consecutive serine residues in *E.coli* (Falco et al., 1985). Table 9 showed composition of purified ALS from yeast and recombinant *E.coli*.

**Table 9: Amino acid composition of three acetolactate synthases**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Saccharomyces cervevisiae ALS</th>
<th><em>E.coli</em> ALS Isozyme II</th>
<th><em>E.coli</em> ALS Isozyme III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>9.4</td>
<td>11.5</td>
<td>9.0</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.4</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>4.7</td>
<td>6.0</td>
<td>4.8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>5.7</td>
<td>4.6</td>
<td>3.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.9</td>
<td>3.3</td>
<td>2.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.8</td>
<td>8.6</td>
<td>8.6</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.3</td>
<td>3.3</td>
<td>3.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.4</td>
<td>3.8</td>
<td>5.3</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.5</td>
<td>3.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.6</td>
<td>10.9</td>
<td>8.8</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.1</td>
<td>3.6</td>
<td>4.1</td>
</tr>
<tr>
<td>Asparagine</td>
<td>4.4</td>
<td>3.1</td>
<td>3.0</td>
</tr>
<tr>
<td>Proline</td>
<td>6.4</td>
<td>5.6</td>
<td>4.9</td>
</tr>
<tr>
<td>Glutamine</td>
<td>4.7</td>
<td>6.2</td>
<td>5.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.4</td>
<td>3.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Serine</td>
<td>6.1</td>
<td>4.0</td>
<td>5.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.3</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Valine</td>
<td>7.8</td>
<td>7.8</td>
<td>9.9</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.0</td>
<td>1.3</td>
<td>2.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.0</td>
<td>2.4</td>
<td>3.2</td>
</tr>
</tbody>
</table>
2.4.5.2 Recombinant systems

*E. coli* is the most commonly used host cell for the over-production of foreign proteins because it is genetically and biochemically well-characterized, and easily handled. The enterobacteria have been used successfully to express ALS from other bacteria (Schloss et al., 1985; DeRossi et al., 1995), as well as active wild-type and mutant ALS from fungi and plants (Poulsen and Stougaard, 1989; Lee et al., 1999; Pang and Duggleby, 1999). In contrast to the bacterial ALS, most eukaryotic enzymes have an N-terminal extension of 60 to 90 residues that functions *in vivo* as an organelle transit peptide. In most cases, the removal of part or all of the transit peptide sequence is crucial for the expressed enzyme to remain in solution and be functional. The specificity and requirements for such processing of the foreign protein in *E. coli* are unknown.

The recombinant ALS gene is very often constructed so as to generate a fusion protein. The fusion partners that are commonly used are the glutathione S-transferase (GST) domain and the hexahistidine (6XHis) tag, usually introduced at the N-terminus. The usefulness of these fusion partners is to greatly improve the recovery of the highly unstable ALS activity since purified protein usually can be obtained in a single chromatographic step. These GST and 6XHis tags can be removed if appropriate protease sites are available, but their presence has been shown not to alter the enzymatic activity (Chang et al., 1997; Hill et al., 1997). In addition, the catalytic and regulatory subunits of bacterial, yeast and plant ALS have been expressed.
independently from each other in *E.coli* (Hill *et al.*, 1997; Pang and Duggleby, 1999).

### 2.4.6 Effect of short chained fatty acid salts on ALS

It has been reported that acetate and propionate induce the synthesis of ALS of *Bacillus subtilis* (Holtzclaw and Chapman, 1974). In a subsequent study (Stormer, 1968; Holtzclaw and Chapman, 1975), the relative effectiveness of most short chained fatty acid salts on the induction of enzyme synthesis was examined. The most effective inducer of the enzyme was isobutyrate and it was about twice as effective as acetate. Propionate at this lower concentration, had only weak inductive effect (Holtzclaw and Chapman, 1975).

**Table 10: Effectiveness of various fatty acid salts at inducing the degradative acetolactate synthase of *B.subtilis* (Holtzclaw and Chapman, 1975)**

<table>
<thead>
<tr>
<th>Medium supplement</th>
<th>Sp. activity of ALS</th>
<th>Relative activity</th>
<th>pH optimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>34.8</td>
<td>1.00</td>
<td>7.5 – 8.0</td>
</tr>
<tr>
<td>Acetate</td>
<td>91.2</td>
<td>2.62</td>
<td>6.5 – 7.0</td>
</tr>
<tr>
<td>Propionate</td>
<td>44.0</td>
<td>1.26</td>
<td>6.5 – 7.0</td>
</tr>
<tr>
<td>Butyrate</td>
<td>40.5</td>
<td>1.16</td>
<td>6.5 – 7.0</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>175.6</td>
<td>5.05</td>
<td>5.5 – 7.0</td>
</tr>
<tr>
<td>Valerate</td>
<td>25.0</td>
<td>0.72</td>
<td>6.5 – 7.0</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>32.0</td>
<td>0.92</td>
<td>6.5 – 7.0</td>
</tr>
<tr>
<td>DL-2-Methyl butyrate</td>
<td>44.0</td>
<td>1.26</td>
<td>7.5 – 8.0</td>
</tr>
</tbody>
</table>

### 2.5 Immobilization

Fermentations traditionally carried out in batch bioreactors employ a variety of freely suspended microorganisms to metabolize nutrients and yield
a number of different fermentation products. The diffusion of substrates including sugars, amino acids, vitamins, and other nutrients into the cell results in their intra-cellular metabolism catalyzed by numerous enzymes. This chain of activities is then followed by the release of such metabolized products out of the cell and back into the bulk liquid medium.

Some authors (Karel et al., 1985; Mensour et al., 1996 and Virkajarvi, 2001) have indicated that the success of large scale industrial systems is dependent on an immobilization system where:

- The carrier material is non-toxic, readily available and affordable.
- The manufacturing system is efficient, easy to operate and gives high yields.
- High cell loading and physical strength are inherent characteristics of the carrier material.
- The cells have a prolonged viability within the support.

Continuous fermentation systems producing fermented product offer, among others, the following advantages over traditional batch systems:

- High volumetric bioreactor productivity (product weight per unit time per unit bioreactor volume).
- Less plant supervision.
- Increased product uniformity.

Immobilization is simply a “tool” used by researchers to contain intact cells bound to an inter carrier within the fermentation vessel. Continuous immobilized cell fermentation systems further boast the following advantages (Mensour, 1996 and Virkajarvi, 2001).
- High cell densities per unit bioreactor volume, resulting in very high fermentation rates.
- The reuse of the same biocatalysis (immobilized cells) for extended periods of time due to constant cell regeneration.
- A continuous process which may be operated beyond the nominal washout rate without losing its entire cell population.
- A discrete phase in which cells may be manipulated.
- Easy separation of biocatalyst from the liquid phase, where the desired products are present, thus minimizing separation costs.
- High cell densities combined with operation at high dilution rates, decreasing the risk of reactor shutdown due to contamination.
- Improved tolerance or protection of cells from inhibitory products.
- Smaller bioreactor volumes which may lower capital costs.

Higher concentration of substrate is inhibitory for butanediol production but high butanediol concentration has little effect on its own production rate (Yu and Saddler, 1982a; fond et al., 1985), therefore to achieve high product concentration in a typical butanediol fermentation process, fed batch approach is particularly beneficial. Yu and Sadder (1983) using this approach carried out butanediol production from glucose at a constant glucose concentration of 20 g/L and obtained AMC+BD concentration of 113 g/L from a total glucose feed of 226 g at a productivity of 0.25 g/L/h.

In a continuous culture, because there is no time gap between inoculation and attainment of metabolic productivity, therefore such a culture results in higher productivity than batch or fed batch cultures (Ramachandran and Goma, 1987). Still higher cell concentration in a typical fermentation
process can be achieved using cell recycle approach which has additional advantages of providing cell free product stream (Ramachandran and Goma, 1988; Qureshi and Cheryan 1989d; Zeng et al., 1991a; Byun, 1994 and Perego et al., 2000). In addition to these techniques, immobilized cell system has been used for butanediol and diacetyl production (Chua et al., 1980 Willetts, 1986; Champluvier et al., 1989; Lee and Maddox, 1986).

Production of butanediol by immobilized cultures has had limited success to date. Batch cultivation of *K. pneumoniae* immobilized in K-carrageenan resulted in the accumulation of 15 gL⁻¹ diol from 50 gL⁻¹ glucose at a productivity of 0.50 gL⁻¹ h⁻¹ (Chua et al., 1980). A 50% improvement in productivity resulted from a switch to a continuous mode of operation, but the resultant yield of diol was only 24% of the theoretical value. Cells of *K. pneumoniae* were immobilized in calcium alginate gel and used in a packed column reactor for the continuous production of BD from whey permeate. A maximum butanediol productivity of 2.2 gL⁻¹ h⁻¹ has been obtained. The system proved to continuous operation (Lee and Maddox, 1985). Moreover techniques have been described for immobilizing *Aeromonas hydrophila* by various methods on butanediol production (Willetts, 1986). Furthermore immobilized intact cells of *Aeromonas hydrophila* by cross linking, attachment to an inorganic matrix, or entrapment in a polymeric gel have been compared. Cells immobilized on a titanium (IV) hydroxide matrix have been suitable for producing 2,3-butanediol from starch, whereas cells entrapped in

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polyacrylamide were a better catalyst for producing the diol from glucose (Willets, 1986).

2.6 Recovery of butanediol

The separation of 2,3-butanediol from the bioprocess medium and subsequent cleaning are the main problems associated with technical scale production. Since butanediol has a high boiling point (180°C), hygroscopicity and the fermented liquors contain proteins, alcohols, polysaccharides, other by-products of fermentation as well as substantial amount of suspended material resulting from deam bacteria (Othmer et al., 1945), it can not be separated easily as a distillate from mash. A number of recovery procedures for butanediol have been reported (Keen and Walker, 1973; Voloch et al., 1985 and Gupta et al., 1998).

In the present scenario, the major part of BD production research is focused on achieving decreased fermentation time for its production. Because 2,3-BD is the product of late stationary phase, since the genes encoding enzymes for its production are expressed only during this phase (Zahler, 1980; Blomqvist et al., 1993 and Renna et al., 1993). So efforts were made to prepone the production of 2,3-BD by manipulating the production of enzyme (α-ALS) by enzyme stimulating agents like fatty acids and fatty acid salts and using mutagenic agents.