## 2.0 LITERATURE REVIEW

2,3- butanediol has been known as a bacterial fermentation product since early part of this century. Shortages of the strategic compound 1,3-butanedine during World War II stimulated intense research efforts in the formation of diol, culminating in the development of pilot-scale operations for both its manufacture and conversion to butadiene. Recent interest in the utilization of renewable agricultural biomass has intensified research on the fermentation of xylose and glucose to 2,3-butanediol. In comparison with fermentation leading to ethanol, lactic acid, acetic acid or acetone-butanol, the conversion of sugars to 2,3-butanediol has attracted less interest during the past. Nevertheless, 2,3-butanediol is an attractive chemical due to its various applications. In fact butanediol is the precursor of a number of compounds. One of these is methyl ethyl ketone (MEK). Compared to ethanol, MEK has a higher heat of combustion, 584.2 vs.326.7 kcal/mol. It also gives an octane number of 96.7 when mixed (25% volume) with gasoline. Thus, MEK is more effective as a liquid fuel additive than ethanol. Apart from this, it is receiving increasing interest not only due to its value as a commodity chemical, but also due to the fact that the process exhibits low product inhibition compared to other solvent fermentation (Magee and Kosaric, 1987, Garg and Jain, 1995).

## 2.1 Earlier Work

Harden and Norris (1906) and Harden and Walpole (1912) carried out the initial work on the production of 2,3-butanediol by microbes. The bacterium employed in these early studies was *Klebsiella pneumoniae*. Donker (1926) is credited with the initial observation of diol accumulation in cultures of *Bacillus polymyxa*, whereas industrial-scale production was first reported by Fulmer et al.(1933). The potentials for using *Bacillus polymyxa* were reported by various authors (Ledingham and Neish,1954; Adams,1946 and Rose,1947) as were those for using *Aerobacter aerogenes* (Pirt and Callow, 1958).

Loube et al. (1984) conducted studies on the production of 2,3-butanediol from xylose by *Bacillus polymyxa* strain 9035 and the best 2,3-butanediol yield was obtained with 1.0% yeast extract. In another experiment, they found that the fermentation of glucose to 2,3-butanediol by *Bacillus polymyxa* was improved by increasing the amount of yeast extract in the culture medium. A level of 1.5%(w/v) resulted in optimal 2,3-butanediol production. They concluded that comparatively high yield could be achieved with 0.5% yeast. Dziewulski et al. (1986) investigated the production of 2,3-butanediol from glucose by *Bacillus polymyxa* in a fed batch mode with biomass recycle. In the fed batch experiment, a medium containing 1% peptone was used with no aeration. The average production of diol per cycle was 65.85mM/100mM glucose utilised.

Fages et al. (1986) investigated the effects of initial sugar concentration and oxygen transfer rate on the production of 2,3-butanediol from Jerusalem artichoke by *Bacillus polymyxa*. They proposed a mathematical model, which uses ATP balance to determine the fraction of substrate fermented to 2,3-butanediol.

Several strains of bacteria and fungi can produce 2,3-butanediol such as *Aeromonas hydrophila* (Willetts, 1985), *Klebsiella oxytoca* (Jansen et al., 1984), *Pseudomonas hydrophila* (Murphy and Stranks, 1951), *Trichoderma harzanium* (Yu et al., 1985) and *Klebsiella pneumoniae*, formerly known as *Aerobacter aerogenes* (Barrett et al., 1983). Chua et. al. (1980) have reported the formation of butanediol by *Enterobacter aerogenes* immobilised on *K.Carrageenan*. They reported maximum butanediol concentration of 11.7mg/ml by immobilised cells after 18 hours of incubation.

Sablyrolles and Goma (1984) have shown that in *Aerobacter aerogenes*, oxygen is a limiting substrate with regard to growth and an inhibitor with regard to specific metabolite productivity. The initial substrate concentration also affects the yields of biomass and butanediol production. The effect of oxygen supply rate and dilution rate on the product output rate and yield of 2,3- butanediol were investigated. The authors have shown that the product yield based on substrate utilised, approached the theoretical value (50%) at low values of

oxygen transfer rate. Jansen et al. (1984) studied the effect of pH, xylose concentration and oxygen transfer rate on the bio-conversion of D-xylose to 2,3-butanediol by *K. oxytoca*. They have developed a model that predicts batch fermentation rates and yields as functions of oxygen transfer and initial specific growth rate of the cells. The model uses bioenergetic principles and material balances. The model was used to predict butanediol yield and conversion rate with respect to oxygen transfer rates.

Papoutsakis and L-Meyer (1984) used the available batch data from literature to work out stoichiometric equations for anaerobic and saccharolytic fermentations of butanediol and mixed acids. The authors emphasized that their equations could be used for checking the consistency of experimental data and for calculating maximum yields. Ramachandran and Goma (1987) studied the kinetics of 2,3-butanediol production from *K. pneumoniae* from glucose using a continuous bioreactor. Their studies indicate that the oxygen transfer rate strongly influences 2,3-butanediol concentration, its yield and productivity. At high dilution rates even though product output remained constant, the product concentration in the effluent decreased.

Lee and Maddox (1984) tested the efficacy of four different microorganisms for producing butanediol from rennet whey permeate. They also conducted

experiments on the continuous production of 2,3-butanediol from whey permeate using *Klebsiella pneumoniae* immobilised in calcium alginate. During a seven-week period of continuous operation, they found a maximum butanediol productivity of 2-3 $gL^{-1}.h^{-1}$ at a dilution rate of 0.77 $h^{-1}$. Qureshi and Cheryan (1989) observed higher cell concentrations and greater 2,3-butanediol production in aerobic cultures of *Klebsiella oxytoca* than with anaerobic cultures. They explained the effect of extensive aeration and agitation on the formation of diol, acetone, acetic acid, ethanol and lactic acid.

Krishnan (1988) studied the kinetics of 2,3-butanediol production from mixed sugar using *Klebsiella oxytoca* NRRL B199. He observed that in a 10% glucose medium at low oxygen levels, butanediol production was favored. In mixed sugar fermentation, as the concentration of xylose increases, diol production decreases, and high levels of dissolved oxygen in this media favours the production of other by-products.

## 2.2 MICROBIOLOGY

Several organisms are known to accumulate butanediol in reasonable quantities, but there is some variation in the stereoisomers produced. Fig. 2.1 shows the three isomeric forms of butanediol, and Table 2.2 summarizes the isomers produced by different organisms. Other bacteria, which are referred to in the literature, include *Klebsiella pneumoniae*, *K. aerogenes*, and *Aerobacter*

*aerogenes,* but these are synonyms for *K.oxytoca* as are *Enterobacter aerogenes* and *E.cloacae.* These organisms are gram-negative, facultative anaerobic rods, and are members of the family Enterobacteriaceae. They produce the L(+)- and meso-stereoisomers in the approximate ratio 1:9. *Aeromonas* (*Pseudomonas*) *hydrophila* is also a gram-negative, facultatively anaerobic rod, but is distinguished from members of the Enterobacteriaceae on the basis of being oxidase-positive and having polar flagella when grown in liquid media.

*Bacillus polymyxa* displays vigorous anaerobic growth in the presence of a fermentable carbohydrate, and may be mistaken for a member of the Enterobacteriaceae, as the Gram reaction is usually negative. *B. subtilis* differs from *B.polymyxa* in that it produces a mixture of the D(-)- and meso-stereoisomers (approximate ratio 3:2), and glycerol rather than ethanol as a by-product. *B. amyloliquefaciens* is similar to *B.subtilis*, but differs in producing fewer by-products (Alam et al., 1990). *B. licheniformis*, although a different species, behaves similarly to *B. polymyxa* except for the production of lactate as a by-product (Raspoet et al.,1991)

**Table 2.1**: Butanediol isomers produced by various bacteria

| Organisms | L (+) | D(- ) | Meso |
|---|---|---|---|
| *Klebsiella oxytoca* | + | - | + |
| *Aeromonas hydrophila* | - | + | - |
| *Bacillus polymyxa* | - | + | - |
| *Bacillus subtilis* | - | + | + |
| *B. amyloliquefaciens* | - | + | + |
| *Serratia marcescens* | - | - | + |

(Source : Voloch et al., 1983)

Although diol formation has been observed in several yeasts (Magee and Kosaric,1987 ; Fields and Richmond, 1967), yields are extremely low. Thus, bacteria are at present the only organisms of industrial importance in the production of butanediol. Key species are found in the genera Klebsiella, Bacillus, Serratia and Pseudomonas. In what follows, each of these organisms has been discussed individually in light of their major characteristics.

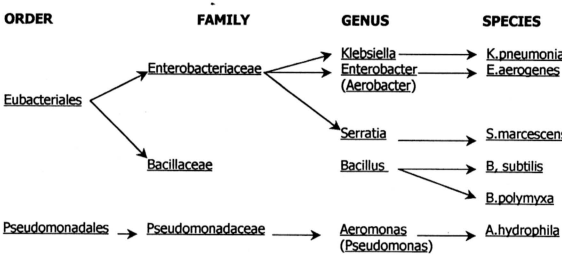



**Fig 2.1. Classification of major butanediol-producing bacteria**
(Source: Buchanan,1974)

Three stereoisomeric forms of 2,3-butanediol may be generated. In any given process, the isomer produced is dependent upon the particular organism employed. This explains the special interest in the utilization of *Bacillus polymyxa* for production of pure levo–diol due to its potential as an antifreeze agent.

### 2.2.1 *Klebsiella pneumoniae* (*Aerobacter aerogens*)

This bacterium, widely distributed in nature, is stable under a wide range of environmental conditions. *K. pnuemoniae* lacks the diastatic properties of *B.polymyxa* but this species typically produces double the amount of diol, with much lower yields of ethanol as by-product (Ledingham and Neish, 1954). The organism also conducts a more complete fermentation, thereby facilitating product recovery. Stock cultures of valuable strains can be stored with ease. For the above reasons, by far the greatest research effort in diol production has focused on the utilisation of this microorganism. In addition, the capacity to produce 2-3 butanediol appears to be widely distributed within the species, so that it is conceivable that strains could be isolated with additional specific advantages.

### 2.2.2 *Bacillus*( Aerobacillus) *polymyxa*

*Bacillus polymyxa* has the ability to ferment a wide range of substrates. Its amylolytic nature enables its use in the conversion of unhydrolyzed grain mashes (Ledingham et al., 1945). Possessing xylanase activity *B. polymyxa* has the potential to fully utilize the hemicellulosic components of natural substrates (Laube et al., 1984 a,b). In this organism, formation of large quantities of ethanol as a by-product is considered to be a disadvantage. Additional problems include loss of fermentation activity due to storage or repeated transfer

(Blackwood et al., 1947) and a susceptibility to attack by bacteriophase (Katznelson, 1944). This organism has the ability to produce pure levo diol.

### 2.2.3 *Bacillus subtilis*

Bacillus subtilis is a second species of the genus Bacillus that has been found to produce butanediol as a major product of the metabolism of carbohydrates. Unlike *B .polymyxa*, however , *B. subtilis* does not produce pure levo diol. Under anaerobic conditions, growth and fermentation rates are low in comparison with other organisms. Vigorous growth together with the formation of butanediol, acetoin, and carbon dioxide, has been observed when oxygen is supplied to the culture (Blackwood et al., 1947). Production of considerable quantities of glycerol, apparently at the expense of ethanol, is a common feature of carbohydrate consumption by this bacterium.

### 2.2.4 *Serratia marcescens*

A member of the same family (Enterobacteriaceae) as *K.Pneumoniae*, *S. marcescens* is also capable of generating reasonable yields of butanediol from monosaccharides. In addition to diol, the formation of significant levels of organic acids (especially formic and lactic acids) typifies these fermentations. Under anaerobic conditions, these two acids account for 37% (molar basis) of the total solvents formed from glucose (Neish et al., 1947). This microbe can utilize both cellobiose and glycerol, but it can not consume arabinose. The ability to ferment xylose is strain dependent. Several other members of genus Serratia

have also identified as diol producers. Chief among these are *Serratia indica, Serratia kiliensis, Serratia plymuthica*, and *Serratia anolium* ( Neish et al., 1948)

### 2.2.5 MORPHOLOGY OF *KLEBSIELLA*

Klebsiella are gram-negative bacilli conforming to the definition of enterobacteria. Members of the Klebsiella group tend to be somewhat short and thick and are straight rods about 1-2μm long and 0.5-0.8 μm wide, with parallel or bulging sides and rounded or slightly pointed ends. The bacilli are either in pairs, from end to end, or are arranged singly. The organisms are killed by moist heat at $55^0$C in half an hour. They may survive in dry conditions for months. When kept at room temperature, culture remains viable for weeks. They are facultative aerobic organisms and growth under strictly anaerobic conditions is poor. The optimum temperature for growth is 37 $^0$C, with lower and upper limits being 12 $^0$C and 43 $^0$C respectively.

## 2.3 BIOCHEMISTRY

The major intermediates in the conversion of pentose or hexose to 2,3-butanediol are shown in Fig. 2.2. The last step in the biological pathway of the fermentation involves the reduction of acetoin (2-hydroxy-2-butanone) to 2,3-butanediol. While 2,3-butanediol has two asymmetrical centers, acetoin has only one, and two sterioisomeric forms, D-(-) and L-(+). Hence, the reduction of

acetoin to 2,3-butanediol may involve as many as two substrates and three products.

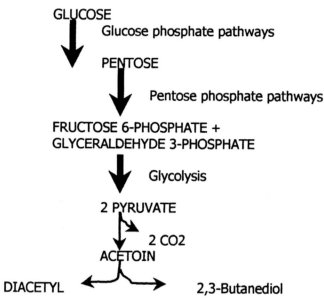



**Fig 2.2. Major intermediates in conversion of a pentose or a hexose to 2,3-butanediol.**
(Heavy arrows represent reactions of the pentose phosphate and glycolytic pathway. Light arrows indicate individual reactions; source: Volvoch et al., 1983).

Most studies have been performed using members of the Enterobacteriaceae, and Fig 2.3 summarizes the mixed acid-2,3-butanediol pathway (Kosaric et al., 1992). Juni (1952) reported that *K. oxytoca* forms acetoin from pyruvate by the action of two enzymes. An acetolactate-forming enzyme catalyzes the condensation of two pyruvate molecules combined with a single decarboxylation to yield acetolactate and $CO_2$. The decarboxylase is specific for the dextrorotatory isomers and the product is the levorotatory isomers of acetoin D-

(-)-acetoin. Both the decarboxylase and acetolactate forming enzymes have been partially purified and characterized (Stormer, 1967; Malthe-Sorenssen and Stormer,1970). The acetoin can be oxidized to 2,3-butanedione (diacetyl by $O_2$ present in the fermentation medium, or enzymatically reduced (with NADH as a co-factor) to 2,3-butanediol.

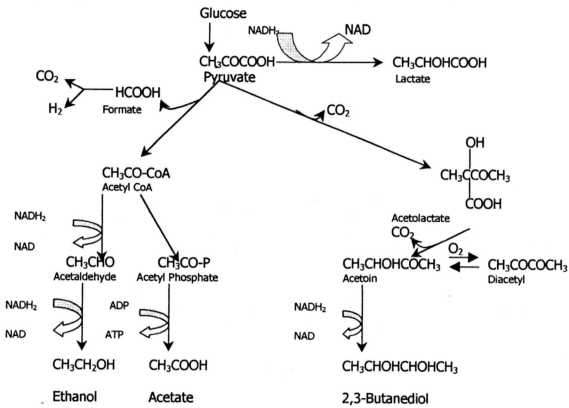



**Fig. 2.3 Metabolic pathway of butanediol production from glucose.**
Source: Kosaric et al. (1992)

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. A variety of organic acids may also be produced. The most common of these are acetate, lactate, formate and succinate. Under aerobic conditions, pyruvate is

broken down to acetyl-CoA in enterobacteria by the action of the pyruvate dehydrogenase multi-enzyme complex. This enzyme system is not synthesized in anaerobic environments, however, and is inhibited by the reduced form of the metabolic cofactor nicotinamide adenine dinucleotide, $NADH_2$ (Gottschalk, 1979).

### 2.3.1 Mechanism of formation of different stereoisomers

The D(-) , L(+) , and meso-isomers of butanediol are all known to be produced by different organisms and the ratio of isomers depends on the organism and the culture conditions. Three models have been postulated to account for the production of different stereoisomers. The first model was developed by Taylor and Juni (1960) whereby, the existence of an acetoin racemase was proposed (Fig. 2.4). The stereochemical reaction product of acetolactate decarboxylase is D(-) acetoin, and it can be reduced to D(-) butanediol by the stereospecific D(-) butanediol dehydrogenase. The stereospecific L(+) butanediol dehydrogenase , on the other hand , converts D(-) acetoin to meso–butanediol. The acetoin racemase converts D (-) to L(+)acetoin to an intermediate, which is then reduced to L(+) butanediol and/or meso butanediol by the L(+) and/or D(-) butanediol dehydrogenase , respectively . This model can account for the different product spectra of different organisms. For example, *K. oxytoca* possesses the acetoin racemase and the L(+) butanediol dehydrogenase, allowing the formation of L(+) and meso butanediol, but not D(-) butanediol . Conversely, some Bacillus species possess the acetoin racemase and the D (-) butanediol dehydrogenase, allowing the formation of D (-) and meso butanediol ,

but not L(+) butanediol . Unfortunately, the flaw in this model is that the presence of racemase has never been demonstrated experimentally. Thus, Hohnbentz and Radler (1978) suggested the following two reasons to account for the various spectra of butanediol isomers.

(1) small amounts of L(+) acetoin are produced by the stereospecific acetolactate decarboxylase,

(2) (2)L(+) acetoin is formed from diacetyl.

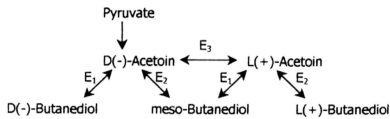



**Fig 2.4 Model for production of different stereoisomers of butanediol. $E_1$, D(-)-dehydrogenase; $E_2$,L(+)-dehydrogenase;$E_3$, acetoin racemase.** (Source:Taylor and Juni; 1960)

The second model was proposed by Ui et al. (1986) for a Bacillus species , and is based on the experimentally proven presence of the enzyme diacetyl reductase (Fig. 2.5). Thus diacetylis formed non-enzymically from acetolactate and/orD(-) acetoin, and is then successively reduced to D(-) acetoin and D(-) butanediol by an NADH-linked D(-) butanedioldehydrogenase. L(+) acetoin is formed from diacetyl by the action of an NADPH-linked diacetyl reductase, but this enzyme cannot reduce acetoin to butanediol. Thus, the D(-) butanediol dehydrogenase reduces L(+) acetoin to meso butanediol. In their experiments, Ui et al. (1986) could not demonstrate the presence of an acetoin or butanediol racemase .

However, Ui et al. (1984) proposed that *K.pneumoniae* does not require the intervention of an acetoin racemase. In this model (Fig. 2.6), three butanediol dehydregenases are postulated so that D(-) acetoin is converted successively to meso butanediol , L(+) acetoin and L(+) butanediol .

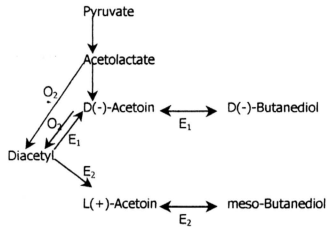



**Fig 2.5. Model proposed for production of different stereoisomers of butanediol by *B. polymyxa.***

$E_1$, NADH-linked D(-)-butanediol dehydrogenase; $E_2$, NADPH-linked diacetyl reductase.
(Source: Ui et al.,1986)

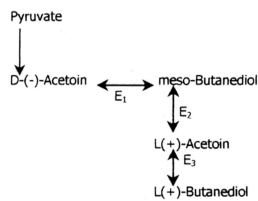
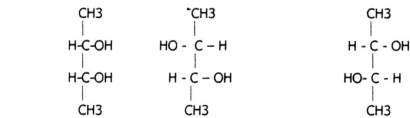



**Fig. 2.6 Model to account for production of different stereoisomers of butanediol by *K. pneumoniae.***

$E_1$, meso-butanediol dehydrgenase (D(-)-acetoin-forming); $E_2$, meso-butanediol dehydrogenase (L(+)-acetoin-forming); E3, L(+)-butanediol dehydrogenase (L(+)-acetoin-forming).
(Source: Ui et al., 1984)

## 2.4 Properties of 2,3-butanediol

2,3-butanediol is a colorless, odorless and viscous liquid. It exists in three stereoisomeric forms of 2,3-BD: D-(-), L-(+) and meso, which are depicted by the conventional planer projection formulas as:



meso-2,3-Butanediol D-( - ) – 2,3-Butanediol L – (+) –2,3-Butanediol

The D and L forms are mirror images and thus are identical in all common physical properties except the direction of rotation of polarized light. Both the meso and ( ± ) racemic forms exist, to a considerable extent. All the three stereoisomers are found in nature in varying proportions, depending on the organism which makes them. Most bacteria give predominantly meso-2,3-butanediol, but the dextro-rotatory and levo-rotatory isomers are also found, particularly the second which is formed to the practical exclusion of all the other isomers by *Bacillus polymyxa*. *A. aerogenes* is reputed to give a small amount of racemic-2,3-butanediol and some of the other species may produce this also.

The boiling points of meso (181-182$^0$C), D (179-180$^0$C) and racemic (177$^0$C) 2,3-BD are slightly different. They all have boiling points much higher than water and thus can not be easily recovered by conventional distillation. The

levo isomer has a low freezing point (-60$^0$C) and its viscosity (0.0218 pa.s) at 35$^0$C is one third that of the meso form. The physical properties of the meso isomer are listed in Table 2.1

**Table 2.2: Physical properties of meso isomer**

| Property | Value |
|---|---|
| Melting point, $^0$C | 23.4 |
| Boiling point, $^0$C | 181-182 |
| Density, g/ml at 25$^0$C | 0.9939 |
| Refractory index at 25$^0$C | 1.4366 |
| Viscosity, centipoises at 35$^0$C | 65.5 |

(Lee and Maddox,1984)

## 2.5 Fermentation process

### 2.5.1 Theoretical Yields

The stoichiometric equation for the reactions leading to butanediol from glucose and xylose are:

Glucose $\longrightarrow$ $2CO_2$ +$NADH_2$ + 2ATP + Butanediol

Xylose $\longrightarrow$ 5/3$CO_2$ + 5/6 $NADH_2$ +5/3 ATP +5/6 Butanediol

Out of the total carbon contained in the sugar, approximately 67% is used up to produce butanediol and 33% is lost as carbon dioxide. On a mass basis, the theoretical yield of butanediol from both glucose and xylose is 0.5. The molar yield from hexose is 1.0 while that from pentose is 0.83.

### 2.5.2 Substrates, Yields, and Productivities

*Klebsiella oxytoca* can ferment a range of sugars including glucose, mannose, galactose, xylose, arabinose, cellobiose, and lactose. *Bacillus polymyxa* can, in

addition, ferment polymeric materials such as xylan, inulin, and starch. Table 2.3 presents a summary of some of the work performed in recent years using free cells in traditional batch fermentation.

**Table 2.3 Summary of some batch fermentation data for various bacterial strains and substrates**

| Substrate | Organism | Overall butanediol productivity(g/l h) | Overall butanediol yield (g/g sugar used) |
|---|---|---|---|
| Glucose | *A. aerogenes* NRRL B199 | 2.02 | 0.45 |
| Glucose | *B.polymyxa* ATCC 12321 | 0.64 | 0.3 |
| Xylose | *K.oxytoca* ATCC 8724 | 1.35 | 0.36 |
| Xylose | *B.polymyxa* NRCC 9035 | 0.1 | 0.24 |
| Mannose | *K.pneumoniae* AU-1-d3 | 0.64 | 0.30 |
| Lactose | *K.oxytoca* ATCC 8724 | 0.86 | 0.21 |
| Starch | *A.hydrophila* NCIB 9240 | 0.17 | 0.2 |
| Starch | *B.polymyxa* DSM356 | | 0.28 |
| Starch, Saccharified | *K.oxytoca* DSM 3539 | 0.66 | 0.5 |
| Molasses,high-test | *K.oxytoca* DSM 3539 | 1.1 | 0.5 |
| Molasses, high-test | *K.oxytoca* DSM 3539 | 1.23 | 0.5 |
| Molasses, blackstrap | *K.oxytoca* DSM 3539 | 0.75 | 0.42 |
| Whey | *B.polymyxa* ATCC1232 | 0.02 | 0.16 |
| Whey permeate | *K.pneumoniae* NCIB8017 | 0.08 | 0.46 |
| Whey | *k.pneunomoniae* ATCC 13882 | 0.38 | |
| Citrus waste | *A.aerogens* | 1.1 | |
| Xylan | *B.polymyxa* NRCC 9035 | 0.02 | |
| Wood hemicellulose hydrolyzate | *K.pneunomoniae* ATCC 8724 | | 0.45 |
| Jerusalem artichoke | *B.polymyxa* ATCC 12321 | 0.79 | 0.4 |
| Agricultural residue | *K.pneunomoniae* ATCC 8724 | | |

(Source: Lee and Maddox, 1984)

It is observed (Table 2.3) that a variety of bacterial strains and substrates have been used, and the fermentation conditions may not necessarily have been optimized. Hence it is difficult to directly compare the results described in the Table 2.3. Much work has been directed towards the fermentation of sugars

present in wood hydrolyzates, particularly those derived from the hemicellulose component. Virtually all of these sugars can be fermented by both *K.oxytoca* and *B.polymyxa*. Given that neither the optimum fermentation conditions nor the most appropriate strains of organisms have necessarily been used, it appears that *K. oxytoca* is the preferred organisms. Unfortunately, this organism possesses neither cellulase nor hemicellulase activity, so a variety of pretreatment methods have been investigated by researchers. For example, Yu et al.(1984c) have described the use of acid hydrolysis as a pretreatment technique. Unfortunately, this technique, and that of steam treatment, can cause the release of water-soluble lignin degradation products, such as vanillyl or syringyl derivatives, which are inhibitory to butanediol production by *K. oxytoca* (Nishikawa et al., 1988a, Frazer and Mc Caskey, 1991). To some extent, the problem can be overcome by prolonged incubation of the cultures. Another approach to wood pretreatment has been that of simultaneous saccharification and fermentation. Culture filtrates from a strain of the cellulolytic fungus *Trichoderma harzianum* have been used to hydrolyze the substrates, while *K. pneumoniae* fermented the liberated sugars (Yu et al., 1984b). The enzymatic hydrolysis was optimal at pH 5.0, but the combined hydrolysis and fermentation was most efficient at pH 6.5. The combined process resulted in butanediol levels that were 30-40% higher than that obtained using separate hydrolysis and fermentation processes.

*Bacillus polymyxa*, the alternative organism to *K.pneunomoniae*, has the advantage of being able to directly utilize polymers, e.g., xylan and starch, but the reactor productivities and yields which have been reported are generally inferior to those of the latter organism. An exception to this may be the use of Jerusalem artichokes (containing a mixture of inulin and sucrose) as a substrate for *B.polymyxa*, where very promising reactor productivities and yields have been recorded. As a substrate for butanediol production, starch has received little attention, probably because of its relatively high cost as a fermentation substrate when compared to agricultural wastes. Work described by Willets (1984) using *Aeromonas hydrophila*, and by Afscar et al.,(1993) using *B.polymyxa*, has demonstrated that prior hydrolysis is not necessary for these organisms, but the productivities and yields obtained were rather low. In contrast, starch which had undergone prior saccharification using a mixture of amylase and amyloglucosidase, proved to be an excellent substrate for *K.oxytoca* (Afschar et al.,1993). High-test molasses has also been reported to be an excellent substrate for *K.oxytoca*, but blackstrap molasses, because of its higher salt content, appears to be less useful (Afschar et al., 1991,1993). Whey, a by-product of the dairy industry, contains lactose and has been studied as a substrate for butanediol production by several groups. Members of the Enterobacteriaceae appear to be more useful organisms than *B.polymyxa*, although one strain of *K. oxytoca* has been reported as producing butanediol only poorly from lactose (Champluvier et al., 1989a). Prior hydrolysis of the lactose using a β–

galactosidase enzyme confers an advantage over the unhydrolyzed substrate (Lee and Maddox, 1984; Champluvier et al., 1989b).

## 2.6 Environmental Parameters

Environmental factors influencing the bacterial production of 2,3-butanediol such as pH, temperature, carbon/nitrogen source, product/substrate concentration, aeration, water activity, medium composition and supplements are discussed in this section.

### 2.6.1 Carbon source

The principal monosaccharides of cellulosic substrates (glucose, mannose, and galactose) as well as major hemicellulosic components (xylose and arabinose), have been used as carbon source for the production of 2,3-butanediol by various microorganisms. The potential for diol manufacture from sugar crops (molasses, sugarbeets, etc) stimulated interest in the bioconversion of sucrose (Pirt and Callow, 1958, 1959). In the absence of oxygen, optimal production of diol was observed for sugars like mannose, galactose, and cellobiose. Under anaerobic conditions, typical yields of butanediol from the batch dissimilation are in the range 0.35 g/g of carbohydrate available (approximately 70% of the theoretical yield). In contrast, yields of acetic acid and ethanol from xylose frequently exceed diol production in the absence of oxygen. Yu and Saddler (1982a) tested the carbohydrates listed in Table 2.4 for diol production under aerobic, anaerobic and finite air conditions (small amount of air initially available in sealed flask) rapidly exhausted as growth occurs. Optimal production of diol was observed in the absence of oxygen for each of mannose, galactose, arabinose, and cellobiose. Biomass formation under anaerobic conditions was lowest when xylose was used as substrate, indicative of the correlation between butanediol and biomass yields.

**TABLE 2.4 : Carbohydrates used in diol production**

| Substrate | | | Solvent yields (g/l) | | | | Diol (+ AMC) | |
|---|---|---|---|---|---|---|---|---|
| Sugar | g/l | Temp (°C) | Diol | AMC | EtOH | HAc | (g/g,CHO available) | Productivity (g/l/hr) |
| Glucose | 20 | 30 | 7.4 | 0.1 | 2.7 | 0.2 | 0.37 | 0.08 |
| Glucose | 10 | 37 | 3.5 | - | 1.8 | 0.1 | 0.35 | 0.14 |
| Xylose | 10 | 37 | 0.4 | - | 1.0 | 0.9 | 0.04 | 0.02 |
| Arabinose | 10 | 37 | 2.5 | - | 1.4 | 0.3 | 0.25 | 0.11 |
| Galactose | 10 | 37 | 2.3 | - | 1.4 | 0.3 | 0.23 | 0.09 |
| Mannose | 10 | 37 | 2.7 | - | 1.5 | 0.3 | 0.27 | 0.11 |
| Cellobiose | 10 | 37 | 1.1 | - | 1.3 | 0.5 | 0.11 | 0.05 |

(Source : Yu and Saddler, 1982a)

The effect of initial sugar concentration on the progress of a batch fermentation process will vary with the composition of the medium. However, it is well established that for synthetic media, where no inhibitory compounds are present, initial sugar concentrations of up to 200 g/liter can be fermented (Jansen et al. 1984a; Qureshi and Cheryan, 1989b; Sablayrolles and Goma, 1984). The decrease in specific growth rate that occurs at sugar concentrations above 20g/liter can be explained by decreasing water activity. Butanediol productivity is much less influenced by the initial sugar concentration than the growth rate, and maximum values occur at a concentration of approximately 100g/liter.



### 2.6.2 Temperature

Optimization of process temperature is recognised as an essential aspect of successful design. The strong dependence of enzymatic activity and cellular maintenance requirements upon temperature (Esener et al., 1983) makes the efficiency of bioprocess strictly temperature dependent. The optimum

temperature for the bacterial formation of 2,3-butanediol is generally reported to be in the range 30-35 $^0$C. Blanco et al. (1984) recorded optimal diol production from glucose at 34 $^0$C. Using sucrose as the carbon source, Pirt and Callow (1958) observed that maximum diol production could be achieved between 35$^0$C and 37 $^0$C. The optimum temperature for sucrose uptake by *K.pneumoniae* was found to be 35 $^0$C. However, production of butanediol is generally accepted to be a growth-associated phenomenon. Thus, conditions for optimal product formation should approximate those of maximum biomass yield. It is not surprising that Esener et al. (1981a), using an Arrhenius-type model, predicted that the maximum specific growth rate for *K. Pneumoniae* should occur at 37 $^0$C. An unexpected result, reported by Laube et al. (1984a), was that diol production by *B.polymyxa* from 1% Xylose was constant at 25, 30, and 35 $^0$C.

### 2.6.3 Nitrogen Source

As a major component of protein, nitrogen must be supplied in large quantities. Hence nitrogenous compounds such as urea, peptone, and yeast extract may be included in the media. Yu and Saddler (1982) reported gains in diol yield from 4% glucose with *K.pneumoniae* upon addition of yeast extract. A 50% improvement in diol yield from 4% xylose was observed when 1% yeast extract was added to cultures of *K.pneumoniae* (Yu and Saddler, 1982). The nitrogen sources tested included urea (1 and 0.5%) ammonium sulfate (0.1 and 0.3%), and cysteine monohydrate (0.2%). It was found that the addition of 1% urea to

a defined medium resulted in a 28% improvement in the yield of diol from 4% glucose (10.49g/l after 2 days). Improvements in xylose conversion by the presence of urea were of similar magnitude. Doubling the ammonium sulfate level of the glucose medium (from 3.0 to 6.0 g/l) resulted in a 16% reduction in diol yield. When the ammonium sulfate content was doubled in a medium containing 4% xylose, diol yields were improved by 45%, resulting in 10.91 g/l diol. In the conversion of glucose by *K.pneumoniae* in the presence of protease-peptone, yeast extract and urea, diol yields were 16.1, 14.6 and 15.1 g/l respectively, from 5% glucose. The optimum nitrogen concentration for diol production was found to be 0.07%.

**2.6.4 pH**

Like temperature, pH is a basic parameter in the regulation of bacterial metabolism. Its influence is especially pronounced in processes characterised by the formation of multiple end products. As a general rule, alkaline conditions favor the formation of organic acids, with a corresponding decline in the yield of such products as butanediol. Predominance of organic acid synthesis was observed under alkaline conditions while diol production is improved by four fold. The optimum pH for diol production appears to be a function of the particular substrate employed. With glucose, the optimum pH is 6.4 (Magee and Kosaric, 1987). In contrast, when xylose is used as the carbon source, diol production is maximum at a pH of 5.0—5.2 (Tsao, 1978).

The pH of the culture is of major importance in butanediol production. Jansen et al. (1984a) used pH control to study the growth of *K.oxytoca* and butanediol production in batch fermentation of xylose. The maximum specific growth rate occurred at pH 5.2 while there was no growth below pH 4.2. The butanediol yield was not strongly affected over the range pH 4.4 to 5.8, but appeared to reach a peak value between pH 5.2 and 5.6. Harrison and Pirt (1967) used *K. aerogens* in chemostat culture with pH control, and demonstrated that at pH 6.2 butanediol production was favored over acetate production, whereas at pH 7.4 the reverse was true. Similar results have subsequently been reported by Schutz et al (1985) and by Zeng et al. (1990a) using *Enterobacter cloacae* and *E. aerogens*, respectively in continuous culture. A similar dependence of butanediol production on pH has been confirmed using *Bacillus licheniformis* (Raspoet et al, 1991) and *B. polymyxa* (Mankad and Nauman, 1992). Thus, it is now well established that maximum yield of butanediol occurs in the range pH 5.0-5.8, while production of acetate is favored at higher pH values. The connection between biomass and diol yield is also evident from pH data. Under aerobic conditions, the maximum specific growth rate (0.61/hour) was observed at pH 5.2 when 10% xylose was the substrate (Jansen et al 1984). No growth was observed when pH was below 4.2.

### 2.6.5 Oxygen

This is perhaps the most important environmental parameter affecting the fermentation process. Although butanediol is a product of anaerobic fermentation, aeration is known to enhance its production. Since butanediol–producing bacteria are facultative anaerobes, they can grow either in presence or in absence of oxygen. Aerobic growth is more efficient. If cells are cultivated in the presence of oxygen, higher concentration of biomass can be achieved. If the oxygen supply is subsequently withdrawn, fermentative metabolism ensues and butanediol production occurs. In this case there is a direct relationship between the volumetric butanediol productivity and the biomass concentration, which was determined by the original oxygen supply. The second reason is that the oxygen supply influences the proportion of various products of the mixed acid-butanediol pathway. This has been illustrated by De Mas et al. (1988). When the supply of oxygen is high, biomass, and acetate and, to a lesser extent, acetoin productions are favored. At intermediate oxygen supply butanediol production is favored, while at a low oxygen supply, lactate and ethanol production predominate. The reason for changes in product proportions is connected with energy supply and the maintenance of correct balance of NAD/NADH within the cell. The situation is complicated by the inhibitory effects of some of the products, like acetate, ethanol, and lactate, on cell growth and butanediol production. In order to optimize the production of butanediol, it is necessary to minimize the production

of by-products, which may be inhibitory. This is achieved by control of the oxygen supply with respect to the demand of the culture. In turn, oxygen supply rate is controlled by the rates of aeration and agitation of the culture (Qureshi and Cheryan, 1989a)

In a batch fermentation of butanediol, there are typically two phases (Jansen et al.,1984a). In the first, the oxygen supply is sufficient to maintain a high value of dissolved oxygen (DO) in the culture. Thus the biomass increases exponentially, but no butanediol is produced. As the biomass concentration increases, the oxygen demand of the culture increases and the DO falls to zero. After oxygen supply becomes limiting (DO< 3% of saturation) the biomass increases linearly rather than exponentially, which means that the growth rate decreases. During the second phase, the fate of the sugar depends on the oxygen supply (controlled by the aeration and agitation rates) and on the oxygen demand of the culture (controlled by the biomass concentration, the culture pH and the presence and absence of growth inhibitors). It has been found that parameters for butanediol are case-specific and need to be determined for a particular bacterial strain, media composition and fermenter design and operation. An interesting approach to the optimization process has been described by Kosaric et al. (1992). He used the redox potential, in conjunction with other parameters such as pH, to monitor culture activity and to maintain optimal growth conditions.

Silveria et al. (1993) has expressed the influence of oxygen in a slightly different way. If the DO of the culture is greater than zero, cell growth is exponential and butanediol production is inversely proportional to the oxygen transfer coefficient ($K_La$). On the other hand, if the DO is zero, growth becomes linear and the maximum specific butanediol production rate is a function of $K_La$. Thus, the maximum specific production rate is dependent on the $K_La$ of the particular fermentation run. Below its maximum, the specific production rate is a function of the specific uptake rate. Similar studies have been performed on the relationship between oxygen supply and butanediol production in B. polymyxa (Fages et al., 1986; De Mas et al., 1988) and comparable conclusions have been drawn.

Since the original report of Harrison and Pirt (1957) demonstrating the importance of oxygen supply in continuous culture, there have been few reports describing this fermentation mode. Zeng et al (1990b) showed that at a particular dilution rate, there was an optimum oxygen uptake rate for butanediol production, and this value increased with dilution rate.

### 2.6.6 Product concentration

High butanediol concentrations influence biomass formation and not product yields. Sablayrolles and Goma (1984) observed that the maximum specific growth rate of *K. Pneumonia* reached an upper limit at a low diol level.

The specific rate of butanediol formation was, in contrast, hardly affected by diol concentrations up to 80 g/l.

### 2.6.7 Effect of other solvents

A study shows that if ethanol is added at 1% level to *K.pneumoniae* cultures growing on 2% glucose, a 45% reduction in yield of diol occurs after 24 hours of growth. This inhibition was however, completely overcome with time (Yu and Saddler, 1982). Addition of 1% acetic acid inhibited diol production significantly (75%), but this too was completely overcome with time. Addition of lower concentrations of acetate was found to enhance diol production.

### 2.6.8 Medium supplements

Culture media must contain all the essential nutrients for the growth and maintenance of the organisms for which they are formulated. In addition, carbon and nitrogen sources, a medium may include vitamins, trace metals, etc.; the selection of which may in part be determined by the nature of the desired fermentation products. In the formation of butanediol, metallic cations have been found to improve conversion efficiencies. Yeast extract contains the cationic species of the following metals: Al, Ba, Cd, Cu, Cr, Fe, Mg, Mn, Mo, Ni, Pb, Ti, V and Zn. Addition of 0.5% yeast extract to the media resulted in improved diol yields (Laube et al., 1984). Phosphate has bee found to stimulate diol production (Murphy et al., 1981a).

### 2.6.9 Inoculum

The size of the inoculum used in bioconversion of either glucose or xylose does not appear to have any significant effect on the final yield of butanediol. With *K.pneumoniae*, the initial fermentation rate for high xylose concentrations was influenced by the inoculum size but the eventual diol yield was not altered (Yu and Saddler, 1982). Adaptation of *K. pneumoniae* to high substrate concentrations prior to inoculation proved effective in the aerobic fermentation of 150g/l xylose (Yu and Saddler,1982).

### 2.6.10 Water Activity

Another important variable affecting 2,3-butanediol production is water activity (which is related to osmotic pressure). The addition of salt to food acts as a preservative by retarding or preventing microbial growth. Lowering of water activity due to increased salt concentration (Jansen and Tsao, 1983) causes growth inhibition. Water activity is an expression of the water concentration that depends on the molar concentration and activity co-efficient of each solute present in an aqueous solution. In organisms such as *K.pneumoniae*, which are known to possess relatively weak osmotic tolerance, water activity may be an extremely important environmental consideration. At a water activity of 0.935, growth of *K. pneumoniae* is reduced to one-half its optimum level.

## 2.7 Culture Techniques

Medium composition has been shown to significantly influence the production of butanediol by bacterial species. The operational conditions of the fermentation vessel are similarly important in the establishment of an optimal process design. Thus the effects on diol yield and productivity of batch versus continuous modes and free cell versus immobilized systems, must be evaluated. Comparing the batch system (Sablayrolles and Goma, 1984) continuous system (Ramachandran and Goma, 1987,1988), it is clear that for almost the same initial substrate concentration, maximum product concentration is obtained only in batch system. But productivity attains a maximum of 9.84 g/L/h in the cell recycle system compared to a productivity of 2.02 or 4.25g/L/h in batch or continuous systems respectively. 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 15g/L diol from 50g/L glucose at a productivity of 0.5g/L (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 from the 25g/l glucose feed at 3.0 g/l/h was only 24% of the theoretical yield. Studies on cells immobilized in activated bagasse by Nigam (1990) have shown that at low dilution rate of 0.04/h, product concentration and productivity achieved were around 20.57 g/l and 0.84g/l/h respectively.

## 2.8 Fed-Batch Fermentation

Fed-batch culture may be defined as a batch culture, which is continuously fed with a growth-limiting substrate. Hence, both the growth rate and the limiting substrate utilization rate are controlled. Classical examples of fed-batch fermentation processes include baker's yeast and penicillin production, where the growth rate and sugar utilization rate are controlled as a means of directing the metabolism of the organism. Other commercial fermentation processes, however, use fed-batch culture not as a means of directing metabolism, but as a means of increasing the total supply of sugar to the fermentation process. This is especially true in processes where a high initial sugar concentration is inhibitory to the growth rate, but the desired metabolic product has little inhibitory effect on its own production rate (Maddox I.S.,1984). Hence the purpose of the fed-batch fermentation process in this situation is as follows.

(1) To increase the final product concentration in the broth, which in turn aids the economics of products recovery, and

(2) to improve the overall reactor productivity by extending the duration of the process and minimizing "downtime". On this basis, production of butanediol is an ideal candidate for the application of fed-batch culture.

Olson and Johnson (1984) demonstrated a fed-batch process using *A.aerogenes* growing on a glucose-based synthetic medium. Initially, the glucose concentration was 100g/l, and after this had decreased to approximately 30 g/L

(fermentation 30h, butanediol concentration 20-25 g/L a continuous feed was commenced. The feed medium contained glucose (450g/L), and nitrogen and phosphate in the same proportion to glucose as in the growth medium. The rate of feeding was such that the glucose concentration in the fermenter was maintained at 30g/L. The results showed that the butanediol production rate was maintained for 108h, allowing a butanediol concentration of 99 g/L, and a glucose utilization of 265g/L, to be attained.

Similar approaches have been taken by Yu and Saddler(1983), who provided the culture with a daily dose of glucose and yeast extract, and by Qureshi and Cheryan (1989b) who added glucose in two steps. Afchar et al.,(1991) developed a pulsed substrate feeding strategy in which the glucose addition was controlled via the carbon dioxide content in the exhaust gas. In all of these examples, the desired aim of obtaining a high product concentration within the fermenter was achieved, thus demonstrating the usefulness of the fed-batch technique. In addition, improved productivities were observed because production was maintained for longer periods of time. However, little attention was paid to the oxygen supply, and it is likely that further improvements could be made if due attention were paid to this parameter. De Mas et al. (1988) recognized this concept during fed-batch culture of *B.polymyxa*. In this case, a constant value of the oxygen transfer rate was chosen to maximize butanediol production and to

minimize acetate production. However, as the fermentation progressed, it became apparent that the oxygen demand decreased while the oxygen transfer rate remained constant. Thus, the higher oxygen availability led to higher levels of acetate. The authors recognized that to maximize butanediol production, the oxygen transfer rate should be progressively decreased during the course of fermentation, to compensate for the decreased oxygen demand.

## 2.9 MOLASSES

The term 'molasses' is applied to the by-product of sugar production, from no significant additional amounts of sucrose can be recovered economically.

There are three principal grades of molasses.

1. Beet molasses is a by -product of the refining of table sugar from sugar beet.
2. Blackstrap molasses is the mother liquor remaining after the crystallisation of brown sugar.
3. High-test molasses, or inverted cane syrup, is crude sugar syrup, which is produced instead of refined sugar. Since it is not a by-product of refining process, its purity is higher than any other types of molasses. Between 60%-70% of the sucrose is converted to invert sugars (glucose and fructose) by treatment with sulphuric acid or invertase enzyme which reduces its tendency to crystalise during concentration and storage. This can result in lower costs since

high-test molasses can be shipped in a more concentrated form than other types of molasses.

In general, beet and cane molasses have comparable amounts of fermentable sugar, potassium, trace minerals, pyridoxine and inositol. Beet molasses can have a five-fold higher organic nitrogen content, but half of it is betadine, which is not assimilate by *Saccharomyces* and other species. Cane molasses is substantially richer in biotin, pantothenic acid, thiamine, magnesium and calcium.

It is apparent from these composition data that molasses is an especially useful media component since it is a good source of nitrogen, inorganic constituents, vitamins as well as carbohydrate. Synthetic media can only support the growth of yeast *Saccharomyces* to a level of 10-20g/L. The replacement of glucose with molasses will produce this yeast in concentrations of 60-100g/L. These nutritional characteristics combined with its economy make molasses one of the most widely utilised raw materials in industrial fermentation media. It is used in the commercial production of antibiotics, yeast, alcohol, and citric acid (Zabreskie, et al., 1980). The most common application of molasses, however, is fermentation of alcohol. Recent developments in this area have been reported by Desantos et al. (1992), Decarvalho et al.(1993) and Donmez and Ozcelik (1992). Desantos et al. (1992) used blackstrap molasses for fermentation of alcohol by yeast. Decarvalho (1993) studied the fermentation in fed-batch mode. Donmez and Ozcelik (1992), on the other hand used beet molasses to ferment alcohol by

Clostridium thermohydrosulfuricam. Monteagudo et al. (1993) used molasses for producing L-lactic acid by Lactobacillus delbrueckii. Park and Baratti (1991) studied the potential of sugar syrup, crystalliser effluent and molasses as for ethanol production by Zymonas mobilis. Afschar et al. (1991) used high-test molasses to produce butanediol. Tayeb et al. (1991) used Egyptian sugar beet molasses, an agrowaste, for production of ethanol.

Depending on the application, it is sometimes necessary to pretreat molasses to reduce suspended solids, salts, $SO_2$, and microorganism content. A typical process mixes the molasses with dilute sulphuric acid to precipitate the calcium salts as $CaSO_4$. The mixture is contacted with steam to raise its temperature and facilitate the precipitation of the $CaSO_4$, strip out the $SO_2$, and pasteurise the mash. Solids removal is affected by centrifugation, after which the mash is cooled and diluted to its final form. In certain fermentations, the mineral content of the molasses is reduced by ion exchange techniques.

**TABLE 2.5 COMPOSITION OF MOLASSES**

| Components | indicative average | Usual range |
|---|---|---|
| Water | 20 | 17-25 |
| Sucrose | 35 | 30-40 |
| Dextrose(Glucose ) | 7 | 4-9 |
| Levulose(Fructose ) | 9 | 5-12 |
| Other reducing substances | 3 | 1-5 |
| Other carbohydrates | 4 | 2-5 |
| Ash | 12 | 7-15 |
| Nitrogenous compounds | 4.5 | 2-6 |
| Non-nitrogenous acids | 5 | 2-8 |

(Source : Zabriskie et al., 1980)