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ABSTRACT

Title of Thesis: Environmental Assessment in Bioethanol Technology Using Immobilized Yeast Cells

Minoo Kandy Zarnegar: Master of Science in Environmental Science and Toxicology, 1988.

Thesis directed by: Sam S. Sofer, Professor Sponsored Chair in Biotechnology.

This study is undertaken from the standpoint of water pollution evaluation in bioethanol technology, using immobilized yeast cells. Evaluation of water pollution caused by immobilized yeast fermentation is the main objective of this research.

The pollution in water remaining at the end of ethanol production, which is normally discharged in the wastewater stream, has been analyzed by the measurement of biological oxygen demand (BOD), since BOD measurement is one of the widely used parameters for water pollution evaluation. BOD obtained from produced water (stillage) was between the range of 115- 360 mg/l. The variation in BOD depended upon the removal of ethanol and concentration of organic matters present in water. Waters with more ethanol and high organic concentration showed higher BOD levels.

This work has demonstrated the following technical accomplishments. A recirculation reactor with total working capacity of 2-3 liters was set up and operated for ethanol production using immobilized yeast cells. This bioeactor has been operated for 37 fermentations using the same yeast cells repeatedly. Amounts ranging from 0.054-0.002 l/g dry yeast of alcohol were produced. Analytical measurement of this alcohol indicated concentration of 2-4% w/v.

Cake obtained after filtration of saccharification slurry, was evaluated as a high-protein supplementary feed for dairy and beef cattle. This product contained 7-8% w/w protein in wet and 28-33% w/w protein in dry state. As a rough estimation, this high-protein feed can be used for 12 days as a complete protein feed or can be supplemented for 36 days with regular feed for growing bulls and Heifer cattle.

Filter cake wash water (1 ml/g cake) was also analyzed for BOD. A range of 360 $\pm$ 10 mg/l was obtained which can be a cause of pollution.

ENVIRONMENTAL ASSESSMENT IN BIOETHANOL TECHNOLOGY USING IMMOBILIZED YEAST CELLS

by
Minoo Kandy Zarnegar

Thesis submitted to the Faculty of the graduate School of the New Jersey Institute of Technology in partial fulfillment of the requirements for the degree of
Master of Science in Environmental Science/Toxicology
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APPROVAL SHEET

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CHAPTER I

INTRODUCTION

1.1. BACKGROUND

The energy crisis has made it necessary to investigate alternate routes for the production of fuel. The fermentation process of agricultural substrates, such as corn, potatoes and molasses into ethanol is an ancient practice that certainly predates to the Egyptian civilization. It was not until the mid 1900's that the microbial production of ethanol was superseded by synthetic ethanol production derived from petroleum.

Central to the operation of an efficient ethanol fermentation process is a proper understanding of the biochemical mechanism of ethanol production. Only with a proper understanding can the many parameters affecting the ethanol fermentation process be optimized for substrate conversion, reduction in production cost and environmental pollution.

1.2. MICROORGANISMS AND ETHANOL PRODUCTION

When microorganisms are grown on sugars in the presence of oxygen, they obtain cellular material and energy

by oxidizing these organic compounds. As a result of this oxidation, the metabolic waste products, carbon dioxide and water are produced. Certain microorganisms are able to grow on organic compounds or other fermentable carbon sources by oxidation.

Microorganisms responsible for ethanol production are facultative, which means they can grow with or without oxygen. If air is allowed to enter the fermentation process in sufficient quantities, then microbial metabolism will switch from an anaerobic, ethanol-producing process to a more efficient aerobic process, halting ethanol production. The previously produced ethanol may actually be utilized and oxidized to carbon dioxide and cell material. Thus, microbes produce ethanol when growth parameters will not support an oxidative metabolic process, thereby requiring facultative microorganisms to employ a less efficient pathway [1,2].

1.3. IMMOBILIZATION AND ITS ADVANTAGES

Immobilization of microbes has been applied to many biochemical reactions. Application of immobilized yeast cells for the production of ethanol has been studied intensively in recent years as immobilization has advantages over conventional free cell systems [3,7]. By immobilizing

the yeast cells in an alginate gel or other matrices, the cell density of the immobilized system can be increased when compared with traditional free cell suspensions in a flow system.

The system facilitates separation and has a great degree of operational flexibility as continuous processes become practical. Immobilized cells can resist high concentrations of inhibitors, such as alcohol and nutrients. In addition, the cell densities of immobilized cells can be much higher than that of the free cells, leading to higher ethanol volumetric productivity [3]. Moreover, immobilized cells can also be dried and stored as a convenient source of reproducible biomass.

In this study, sodium alginate was used for the immobilization of yeast cells. Alginic acid is a natural polysaccharide obtained from extracts of sea weed. The structure and molecular weight of alginate depends on the type, age, and part of the sea weeds used and also on the extraction process [6].

1.4. MASS TRANSFER CHARACTERISTICS

It is important to understand the mass transfer characteristics of the substrate and products within the calcium alginate matrix. Mass transfer through the alginate

gel is a critical parameter that affects the kinetics of an immobilized biocatalyst. The mass transfer characteristics of calcium alginate gel depend on the grade of alginate used, the concentration of calcium ions in the curing solution and the hydrodynamic properties of the fluid surrounding the yeast cells [3,6,7].

The grade, concentration and diffusion characteristics of the alginate gel are reflected by changes in the diameter of the bead. Large pore size can cause problems by allowing the entrapped enzymes or cells to leak out. Polymeric gels like polyvinyl alcohol have smaller pore size and can be used to overcome the leakage of cells or enzymes, but they offer more resistance to the diffusion of substrates. It is reported that calcium alginate gel provides little barrier to the diffusion of substrates up to a molecular weight of 5,000 [7].

Mass transfer resistance around the outside of the beads may reduce the effectiveness of microbial activity per unit volume by limiting the availability of substrates resulting in lower specific substrate utilization. According to some studies [7], mass transfer resistance around the beads is closely related to system parameters used in the reactor, such as flow rate, bead size and bead composition, but mass transfer within the beads is still limited by intraparticle diffusional resistance.

1.5. REACTOR DESIGN

Due to economic considerations, improvements in sethanol fermentation have focused on increasing the productivity of the fermenter, while maintaining high ethanol concentrations. Since the metabolic conversion of sugar to ethanol is governed by the microorganism, high cell densities in the fermenter improve the productivity. In standard continuous industrial fermenters, however, a fraction of the biocatalyst is lost in the effluent, thus reducing its concentration [4]. Special reactor configurations have been devised to prevent such losses. The most important of these types are cell recycle reactors and immobilized cell reactors. While cell recycling requires additional equipment, such as settlers or centrifuges, immobilized cell reactors are a less costly and less complex.

The recirculation reactor has been described by Chambers et al. [27]. It is a tool designed to determine a flow regime that minimizes mass transfer resistance. In a high mass transfer resistance flow regime, the substrate concentration at the surface of the immobilized yeast beads is lower than the bulk concentration because of external resistance to mass transfer. As the flow rate is increased, the reaction rate increases until a plateau is reached,

indicating that the resistance to external mass transfer has been overcome and the concentration of substrate at the surface is essentially equal to the bulk substrate concentration. Based on this principle, the performance of an immobilized cell, recirculation reactor is studied for ethanol production.

1.6. WATER POLLUTION IN BIOETHANOL TECHNOLOGY

Interference with the operation of wastewater treatment systems is caused by a wide variety of chemical, biological and physical phenomena. In a broad sense, interference consists not only of material which inhibits biological sewage treatment processes, but also substances which cause problems in sewage collection systems, sludge disposal or utilization methods and land application of wastewater [30].

Wastewater is the flow of used water from a plant or community, which contains 99.94 percent water by weight. The rest (0.06 percent) is material dissolved or suspended in the water. The suspended matter is different from pollutant in solution. Industrial wastewaters from the ethanol production industry, on the other hand, may consist of large volumes of water used in processing industrial products [30].

The wastewater components of major concern are those which deplete the oxygen resources of a stream or a lake into which they are discharged. However, a pretreatment policy is essentially dictated by the federal regulations that have been promulgated to date. Pretreatment may be necessary for compatible or incompatible pollutants. Both may be limited in the National Pollutant Discharge limination System (NPDES) permit.

Based on this information, water pollution was evaluated in bioethanol technology by the determination of biochemical oxygen demand (BOD) in stillage (bottom residue after ethanol distillation).

1.7. RECYCLING OF PROCESS WATER

Recycling of produced water has many advantages in different industrial plants in food processing technology. Recycling and reusing of water can reduce process water consumption, lowering the water pollution and cost of pollution.

Recycling of fermentation liquid (glucose water) through the immobilized cell reactor is different from that of the free cell recycle reactor, since spent fermentation liquid from the ICR contains residual nutrients and enzymes,but not free cells. However, studies [20] have

shown that total recycling may not be desirable because unwanted materials build up and poison the fermentation broth.

For example, acetic acid as an alcoholic by-product, because of its low volatility, can remain in stillage (bottoms from alcoholic distillation) and cause an acidic condition in recycled water. This can interfere with an ideal recycle because it damages enzyme activities and also causes corrosion in sewer systems and wastewater treatment plants. However, this acidic material can be removed by physical and chemical reaction to prevent accumulation in the fermenter [36]. Alternatively, it could be metabolized by a special strain or mixed culture or neutralized by adding caustic reagents, such as calcium carbonate or sodium carbonate.

CHAPTER II

RESEARCH OBJECTIVE

The main objective of this research is the evaluation of pollution in bio-ethanol technology, using immobilized yeast cells in a recirculation reactor. Primarily, a recirculation reactor is set up and the effects of environmental characteristics such as pH, temperature, flow rate, nutrient and substrate concentration of ethanol production studied. A certain amount of yeast is immobilized in alginate gel for several repeated fermentation experiments, for the estimation of bead viability and productivity. Different amounts of yeast are immobilized and used in fermentation for obtaining the most effective concentration of dry biomass in ethanol productivity.

Preparation of high-protein food from filtration residues which remain in the filter after separation of glucose is a research objective. Protein content of this product is evaluated and its value as supplementary feed for dairy and beef cattle estimated.

This high protein feed is an important supplement as a substitute for growth stimulating products such as hormones.

From the standpoint of pollution, water pollution is investigated by measurement of biochemical oxygen demand (BOD). BOD testing is a main parameter for water and wastewater pollution evaluation. An attempt is made for recycling the produced water after separation of alcohol from water. It is found out how recirculation may affect pollution reduction, process water consumption and cost of production and operation in bio-ethanol technology.

CHAPTER III

LITERATURE REVIEW

Pauline M. Doran et al. [4], investigated the effects of immobilization on the growth and fermentation properties of Saccharomyces cerevisiae. Their experimental evidence on altered metabolism in immobilized cells suggests that immobilization can affect the biochemistry of cell function and growth. Many studies have indicated that a wide variety of modifications may also occur in cells after immobilization. For example, conditions for optimal growth are often different from those for suspended cells, while product yields and lag times may be affected. There have been many claims that immobilization causes activation of the cell metabolism. While some careful studies have distinguished that the effects of cell proliferation cause a real enhancement in metabolic rate, reports of increased productivity with immobilized cells should be examined cautiously.

In another study Tanaka et al. [26], studied diffusional characteristics of several substrates of varying molecular sizes into and from calcium alginate gel beads. It was found that diffusion of higher molecular weight substrates was limited more strongly by the

increase of sodium alginate concentration in the gel beads, than by the increase in calcium chloride concentration used in curing the beads. Substrates having molecular weight less than 20,000 were easily taken into the gel.

In another study, Gosmann and Rehm [28] investigated the oxygen uptake of three different microorganisms; Pseudomonas putida, Saccharomyces cerevisae and Aspergillus niger, all immobilized in calcium alginate gel. The oxygen uptake was compared with respiration of free cells. It was shown that the specific oxygen uptake of free microorganisms decreased at lower cell concentration. On the other hand, by increasing cell concentration in the gel, oxygen was consumed faster that it could diffuse into the beads. At this point, the cells had to compete for oxygen, and diffusion became the limiting factor for oxygen uptake.

Chien and Sofer [3] also studied the performance of immobilized yeast cell reactors. They studied a number of parameters including flow rate, amount and growth rate of yeast, bead size and type of medium. They showed that variation in these parameters had a pronounced effect on the fermentation rate. The paper presents typical ranges of the above parameters and the patterns of changes

that take place when bead size and reaction medium are varied. Saccharomyces cerevisae cells were immobilized in calcium alginate beads for the production of ethanol. Different flow rates and bead sizes were used to optimize the productivity. The productivity of 3.34 mm beads at a flow rate of 8.8 liter/hr was 95 percent higher than that at 1.0 liter/hr. Maximum productivities of 0.34, 0.27 and 0.22 g ethanol/g yeast/hr were obtained for 9.2 percent yeast and immobilized beads of 3.34, 4.45 and 5.56 mm in diameter, respectively. These compares with maximum rates of 0.30 for free cells of equivalent cell density.

In an investigation by P.M. Doran and co-workers [4], the kinetic properties of Saccharomyces cerevisiae immobilized on crosslinked gelatin were found to be substantially different from those of the suspended yeast. Batch fermentation experiments conducted in a gradientless reaction system allowed comparison of immobilized cell and suspended cell performance. The specific rate of ethanol production by the immobilized cells was 40-50% greater than for the suspended yeast. The immobilized cells consumed glucose twice as fast as the suspended cells, but their specific growth rate was reduced by 45%. Yields of biomass from the immobilized cell population were lower at one-third the value for the suspended cells. Cellular

composition was also affected by immobilization. The measurement of intracellular polysaccharide levels showed that the immobilized yeast stored larger quantities of reserve carbohydrates and contained more structural polysaccharide than did suspended cells. Flow cytometry was used to obtain DNA, RNA, and protein frequency for immobilized and suspended cell populations. Data of this study showed that the immobilized cells have a higher ploidy than cells in suspension.

In another study, J.L. Vega and colleagues [8] found that acetic acid, a by-product of ethanol fermentation by Saccharomyces cerevisiae, has been shown to inhibit cell growth if present in high concentration. Consequently, acetate has been considered undesirable in systems where the production rate depends upon steady state growth. Acetate, however, may be desirable in some systems, since it increases the maintenance requirements of the yeast. In immobilized cell reactors using the crosslinking method, steady state is not achieved and cell overgrowth becomes a problem. They also studied how they can take advantage of using acetate, both to reduce cell overgrowth and to increase ethanol productivity. Various concentrations of acetate were added to batch and plug flow systems, while monitoring the effects on cell growth and ethanol

production. The productivity was increased by as much as 50 percent in an immobilized cell reactor (ICR), while cell growth was greatly reduced.

Previously, Douglas B. Rivers et al. [31] studied effects of potential waste streams resulting from ethanol production by the simultaneous saccharification fermentation (SSF) of cellulose, determined by measuring the biochemical oxygen demand (BOD). According to their studies a worst-case analysis of (BOD) from ethanol-containing beer, showed an initial value of 1670 mg/l BOD which is 29% higher than projected initial BOD and 32% greater than the projected waste-discharge. They found that when ethanol was reduced to 0.1-0.2% w/v, BOD levels were 605 mg/l in mash and 250 mg/l in beer. Both values were well below the projected discharged levels.

They investigated the effects of high concentration of ethanol and solids such as saccharification mash on BOD level. Since solids may always be removed from the mash by filtration for future use, ethanol would not be dumped in a waste stream unless the recovery was not economical. The average BOD level in their study shows a linear relationship to ethanol concentration. It means the higher the ethanol concentration, the more BOD. Finally, when ethanol was distilled and removed, the resulting BOD indicated a

decrease as ethanol was removed. At ethanol depletion, the average BOD was 605 mg/l while at total removal it was 450 mg/l.

CHAPTER IV

EXPERIMENTAL APPARATUS AND PROCEDURES

4.1. MICROORGANISMS SELECTION

Although numerous microorganisms are capable of producing ethanol, not all are suitable for industrial processes. Yeast cultures, in particular Saccharomyces cerevisae sp. have been most extensively examined for ethanol production processes because they are very efficient in converting sugar into ethanol and are not as strongly inhibited by high ethanol concentrations, as are other microbes. Yeast's cellular dimension generally ranges from 2.5 to 10.5 microns in width and 4.5 to 21 microns in length [22]. In this investigation, Distillers'active dry yeast, from the Red Star Company was used. Theoretically, it has been indicated that two moles of ethanol can be produced from one mole of glucose (511 Kg of ethanol from 1000 Kg of glucose) [2].

4.2. IMMOBILIZATION

The entrapment of microorganisms in calcium alginate gel was conducted as follows. Distilled water, 100 g active dry yeast and 5 g sodium chloride (NaCl) were used. After

dry yeast and 5 g sodium chloride (NaCl) were used. After soaking yeast in part of the distilled water and sodium chloride in a Hamilton Beach blender, it was mixed well for a few seconds. Then 7.5 grams sodium alginate and the rest of the distilled water were added to the mixture very slowly, to allow the entrapment of cells by alginate gel.

While alginate gel is being added, the mixture is stirred continuously until a homogeneous cell suspension is formed. Mixing time must be as short as possible because excess mixing may damage the cells physically. With the help of a syringe pump (Sage Instrument, Model 351), the homogeneous cell suspension is then extruded as discrete droplets in a slowly stirred 0.1M calcium chloride solution. On contact with calcium chloride, the droplets harden to form beads about 3 to 3.5 mm in diameter. Here, calcium chloride acts as a cross-linking agent. The beads are then cured in calcium chloride for 24 hours at $4^{o}C$ before use. For the preparation of the beads, a bead maker sieve may also used, but the beads formed are bigger in size and with less uniform shapes when compared to the syringe pump beads.

4.3. SUBSTRATE PREPARATION

Microorganisms can metabolize anaerobically a wide variety of organic compounds and produce ethanol. These

substrates may be derived from agricultural commodities such as corn, potatoes, sugar cane, sugar grains, or any other crop high in starch or sugar. In this study, cornmeal was used as a source of substrate for fermentation. Cornmeal was supplied from Goya Food Company in New Jersey. The production of ethanol from cornmeal requires degradation (hydrolysis) of its starch content. The fermentable sugar, such as glucose and maltose can then be metabolized to yield ethanol.

Starch is a dense, water insoluble granule which is resistant to enzyme attack in its native form. Before starch can be processed with enzymes, it must be dispersed in solution and rendered susceptible to breakdown. Starch solubilization is accomplished with heat (gelatinization). Once it is gelatinized, starch molecules are broken down by the enzyme Taka-Therm or Taka-Lite (Miles Laboratories), to a soluble dextrin hydrolysate that has a viscosity suitable for further processing (liquefaction).

In this experiment, 700 g cornmeal was mixed in 3 liters of water, in a ratio of 1:4 or 23% w/v, in the cooking pot. The slurry was heated to 60^{o}c and an enzyme Taka-Therm was added, as heat continued to reach 90^{o}c. This temperature is an optimum point for this enzyme during a one hour time period, to permit the efficient liquefaction of

starch. After this point, heating is stopped and the slurry is cooled to around 60°c for the addition of the enzyme, Diazyme (Miles). This helps all the dextrin and maltose to be converted to glucose.

The following reaction indicates the overall process for the glucose production:

$$(C_6H_{10}O_5)_n + (H_2O)_n \xrightarrow[90^{o}c]{\text{Taka-Therm}} \text{Dextrin} + \text{Glucose} + \text{Maltose}$$

One Hour

$$\text{Dextrin} + C_{12}H_{22}O_{11} \xrightarrow[60^{o}c]{\text{Diazyme}} n(C_6H_{12}O_6)$$

(Maltose) (Glucose)

Once the hydrolysis process of starch is completed, the slurry is then passed through a rotary filter for the separation of glucose water and filter cake. The glucose water is transferred to a recirculation bioreactor for the fermentation process, while the filter cake is either dried from 45-50 weight percent water to 10-12 weight percent water using an oven, or is frozen and stored for further analysis.

4.4. ETHANOL PRODUCTION IN A RECIRCULATION REACTOR

4.4.1. REACTOR SET UP AND OPERATION

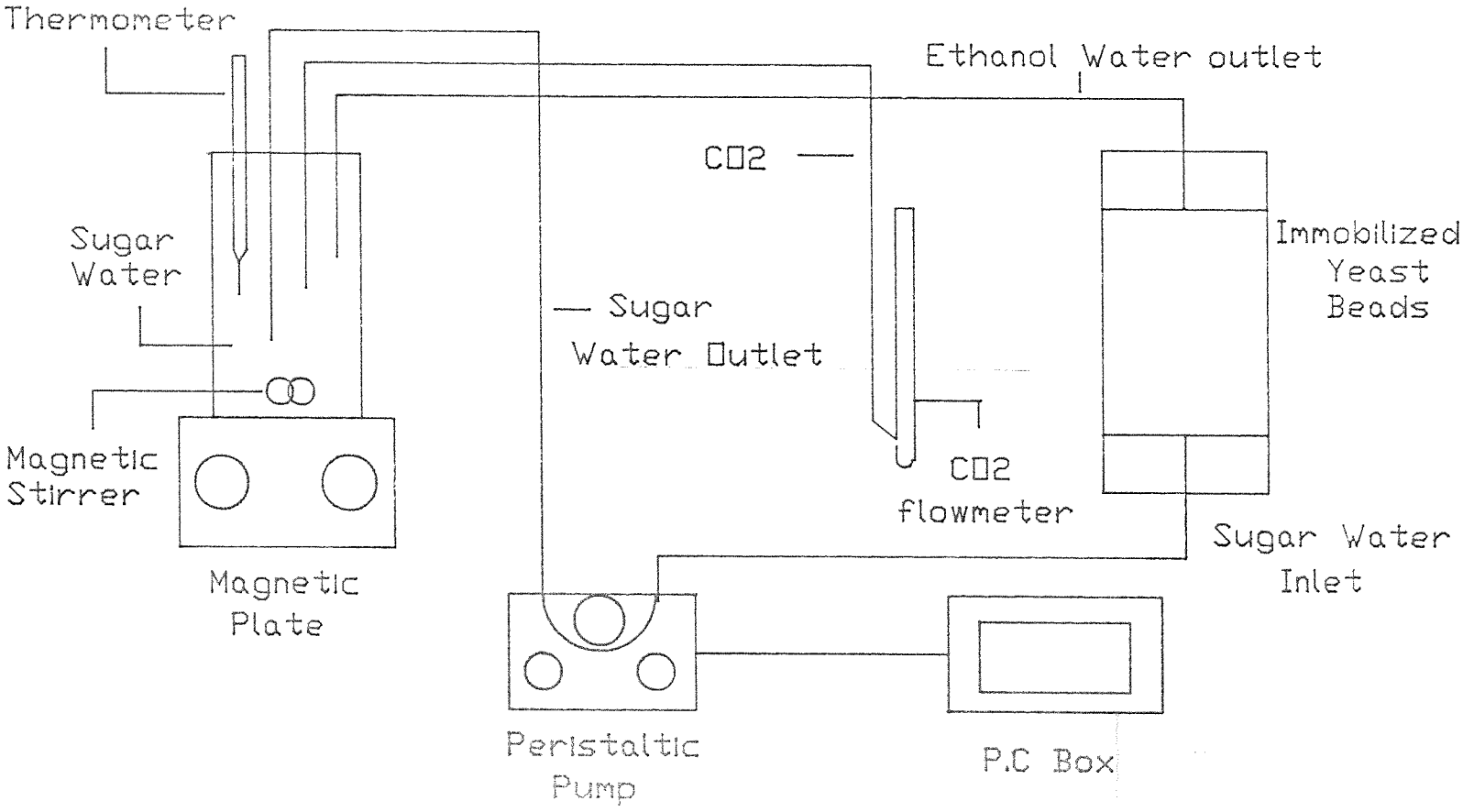


Fig.1: Recirculation Fermentation Reactor

A recirculation reactor was set up for continuous alcohol production using immobilized cells. Figure 1 shows a schematic configuration of this experimental fermentation reactor. The system is composed of a reservoir, peristaltic pump, immobilized cell reactor, gas flow meter and a sample port. The reservoir has a capacity of 3 liters with a working volume of 1.8 to 2.5 liters. The immobilized cell reactor has a capacity of one liter, 6.2 cm in length, 3.1 cm OD, and 3 cm ID in diameter. The working volume of this reactor, however, is 400 ml or lower. The total working volume is 2 to 3 liters. The reaction medium is constantly mixed by a magnetic stirring bar. A 21 gauge needle through the center of a size 2 rubber stopper is used as the sample port. Substrate is circulated with the aid of a peristaltic pump through the immobilized cell reactor (ICR). The reaction temperature is maintained at 25 to 30^{o}C and pH in the range of 3.5 to 5, which is adjusted by an acidic compound such as hydrochloric acid if necessary.

In the ICR, alginate yeast beads are held in between two adjacent screens, so that the beads will not float to the top of the reactor and form a dense packing that could lead to fluid channeling and also block the release of CO_2 gas. Calcium alginate yeast beads are washed before each use so that calcium chloride may be removed. The reaction

medium consists of 10 wt % glucose, and is maintained at this concentration throughout all experimental runs. A complete nutrient medium is added to the reaction substrate when the yeast activity is reduced.

The nutrient medium is composed of :

Glucose.....20 g/l

KH_2PO_4.....5 g/l

$(NH_4)_2SO_4$.....2 g/l

$MgSO_4 \cdot 7H_2O$.....0.4 g/l

$CaCl_2$.....0.1 g/l

Yeast extract (Difco).....2 g/l

Before the start of each experiment, the reactor was first washed with methanol and then with hot distilled water for 2 to 3 times until the tubes, reservoir and immobilized cell reactor (ICR) were all sterilized. This was done because contamination of the reactor causes interference of other microorganisms with the yeast's cells activities.

Three experimental runs were performed. Operational parameters and reactor set-up were the same throughout all three runs with the exception of the flow rate, and yeast cell dry biomass. Also run # 3 was controlled by a computer. Operational parameters are summarized in Table 1.

Table 1. Operation Parameters

Temperature	25-30°C
pH	3.5-5.0
Glucose concentration	10% w/v
Glucose water volume	1.8-2.5 L
Flow rate:	
Run 1	414 ml/min
Run 2	660 ml/min
Run 3	330 ml/min
Biomass concentration:	
Run 1	55.0 g
Run 2	36.0 g
Run 3	32.0 g
Bead size:	
Run 1	3.5-4.4 mm
Run 2	3.0-3.6 mm
Run 3	3.0-3.6 mm

Reactor working volume was maintained between 2 to 3 liters at all times.

Run # 1 was conducted 37 times with one batch of yeast cell beads. The size of the beads were 3.5 to 4.4 mm in diameter and each bead weighed approximately 0.194 g in dry yeast. Total weight of the batch was 55 g dry yeast biomass and 350 g wet beads.

Run # 2 was carried out 20 times with the same conditions as the first run, except with smaller beads, 3 to 3.6 mm, with 0.156 g dry yeast in each bead, less amount of wet beads(250 g) were used to evaluate mass loading in the reactor. In these two experimental run, the period of each experiment was 6 to 8 hours, averaging of 7 hours. Each experiment was stopped at the end of total conversion of glucose to ethanol and carbon dioxide. Beads were washed and stored in 0.1M calcium chloride solution until next use. Ethanol water was collected for the separation of ethanol using a distillation kit. A simple distillation kit with a fractionating column was used. Once ethanol and water were separated, ethanol was analyzed for concentration and water was analyzed for pollution evaluation and/or recycling.

Run # 3 , as was previously mentioned, was controlled by a continuously working computer. This run was split into 3 intervals, where each time the reactor worked for 5 days continuously. An addition of glucose to the

substrate reservoir, however, was needed every 8 to 10 hours.

It is noteworthy to mention that in run # 1, a nutrient medium was added anaerobically twice, on day 17 and day 27, for strenghtening of the yeast cell activity and in run # 2 a nutrient was added once, in the presence of oxygen.

4.4.2. ANALYTICAL METHODS

A gas flow meter set on the reactor was used to monitor periodically carbon dioxide generated from the fermentation reaction at room temperature(25-30 ^{o}c).

Samples of ethanol water were taken periodically, using a sample port set on the reactor. These samples and also ethanol separated from the ethanol water were analyzed on a gas chromatograph(Varian GC 3300) and by a refractometer, which is a less sophisticated instrument these days but an accurate one for the measurement of alcohol concentration.

GC conditions are as follows:

Injection volume.....1 microliter

Injection port temperature.....140^{o}c

Column temperature .....120^{o}c

Detector temperature.....200^{o}c

Range 9.0

Analysis time .....5.0 minutes

Ethanol retention time .....0.28 minutes

Column .....Alltech #8011/2, SS, 6'chrom. W-HP

Calibration .....Plot integrated area versus known concentration of ethanol

4.4.3. CHECK FOR VIABILITY OF BEADS

Viability of immobilized yeast cells is done in a microassay bioreactor. Viability is defined here as the ability of microorganisms to consume dissolved oxygen from the medium for endogenous respiration. The medium used is distilled water, instead of glucose. The experimental set-up is shown in Figure 2.

The microassay reactor consists of a 1.9 ml jacketed reaction vessel, with a small magnetic stirring bar. The concentration of dissolved oxygen was monitored using a Clark-type dissolved oxygen probe. Water at the required temperature (37^{o}c) was circulated in the jacket through a water batch (Haake, Type-439). The reactor was mounted on a stirrer plate, and the magnetic stirrer bar maintained uniform oxygen concentration. The oxygen probe was connected through an amplifier to a chart recorder assembly

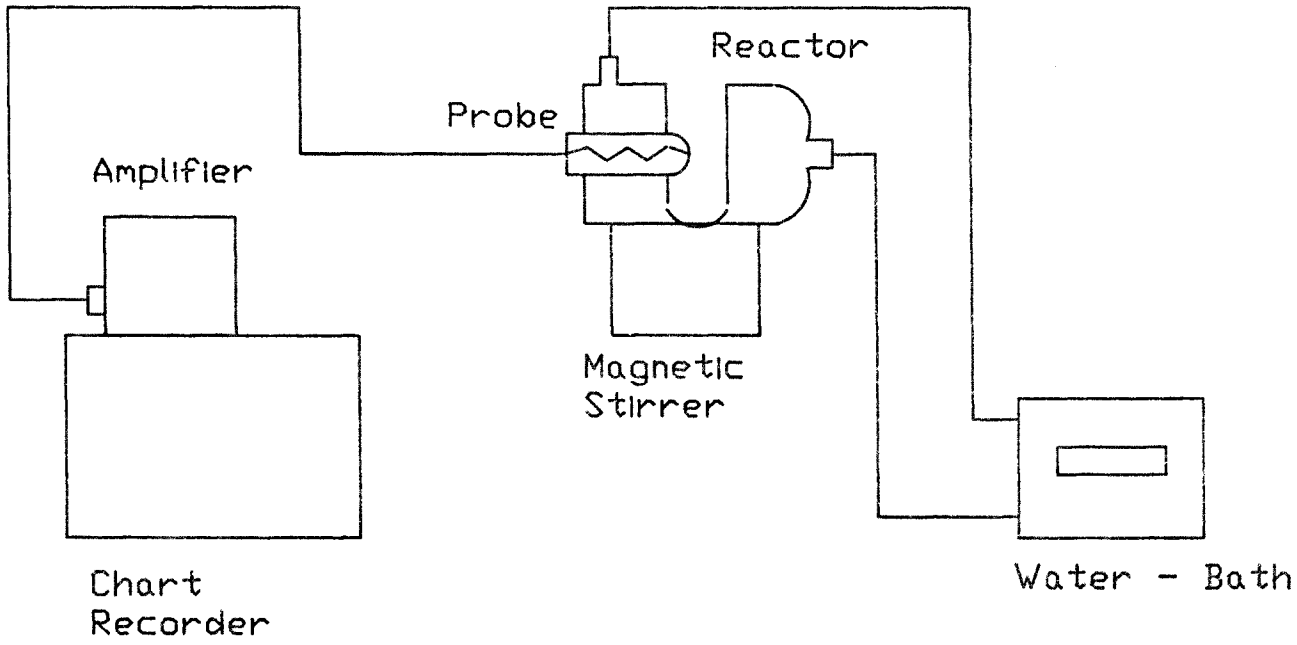


Fig. 2. Experimental Setup of Micro-Assay Reactor.

(Schlumberger, Model EU-205-11), which recorded changes in the dissolved oxygen concentration.

Before the start of each run, the reactor was sterilized in an autoclave at 120°c and then washed successively, with methanol and distilled water several times.

Distilled water(1.6 ml) was then added to the reactor and saturated with oxygen by bubbling air through it. The saturation concentration of oxygen from air in water at 37°C, was estimated to be 200 nmole/ml. After saturation with oxygen, 2 to 3 beads weighing a total of 0.1 to 0.12 (± 3.0 percent) were shocked at 42°c for 2 to 3 minutes in distilled water and then put into the reactor. The shock treatment was carried out to revive the microorganisms from their dormant state. The reactor was then sealed from the top, and the concentration of dissolved oxygen monitored on the strip chart recorder. Sufficient mixing was allowed to overcome any mass transfer resistance.

4.4.4. CELL GROWTH AND DENSITY

Throughout all three runs for ethanol production, the operational parameters (pH, temperature and substrate concentration) were maintained in a desirable condition for yeast cell activity. Periodic samples of beads were taken

for ethanol analysis, viability measurements and stability of beads. Concentration of cells were determined by the measurement of protein, following a total protein procedure obtained from Sigma Chemical Company, using the Lowry method.

Principally, the Lowry reagent removed interfering substances such as Tris, sucrose, salts, etc. from the sample. The Foline and Ciocalteus phenol reagent reacts with peptide bonds of protein molecules and a purple-blue complex develops. The dye intensity is measured at 600 nanometers, which is proportional to the total protein or enzyme content of the cell. The higher the intensity, higher the protein and the larger the cell size.

Experiments are conducted as follows:

1. Sample of beads 0.2 to 0.5 g wet wt. is taken randomly, and placed in test tube.
2. 5 ml distilled water is then added to the tube.
3. Alginate gel coating is dissolved by mashing the beads using a round bottom glass rod, until a uniform and homogeneous solution is obtained.
4. 1 ml of this solution is transferred into a small volumetric flask with a desirable volume (100, 50, or 25 ml), the sample is diluted with distilled water to the flask's volume.

5. 1 ml of this well-mixed solution is then pippeted into a test tube.
6. 1 ml Lowry reagent is added to this tube and left at room temperature for 30 minutes, so that interferences are removed.
7. 0.5 ml Foline and Ciocalteus reagent is added to the tube and there is waiting period of 20 minutes for peptide- foline complex to be formed.
8. Intensity of dye formed is measured on a Bausch and Laumb Spectronic 20 spectrophotometer, set at 600 nm. A blank is also required to be prepared.
9. Concentration of protein is obtained using a calibration curve.

Protein content of the cake after filtration of saccharification slurry was measured using the same procedure.

The number of reused cells were also counted. A sample of beads 0.2 to 0.5 g wet wt. were placed in a vial with 5 ml of 20% w/v sodium pyrophosphate solution for the separation of alginate gel support. After all the alginate gel support is dissolved, the cell containing liquid is then transferred in a tube and alginate gel is washed out 2 to 3 times with distilled water. A 10^{-1} dilution was made using 0.5 ml of mixed cell liquor, pippeted into a 4.5 ml of 0.1%

tween solution. A series of dilutions (10^{-3} to 10^{-5}) were made with sterile distilled water. Selected dilutions (10^{-3} to 10^{-5}) were used for yeast cell productivity. Yeast cell productivity was observed microscopically. The number of cells were counted, using a lined chamber.

4.5. ASSESSMENT OF POLLUTION

In regard to the pollution, water pollution was a main consideration in this study. Pollution of water was measured by determination of the biological oxygen demand (BOD)test. Total suspended solids were also measured.

4.5.1. BIOLOGICAL OXYGEN DEMAND (BOD)

The BOD was determined in accordance with method 507 of the Standard Method for the Examination of Water and Wastewater [31]. The samples analyzed in this study, are mainly stillage (bottoms from alcohol distillation). Samples were collected and cooled to 20°C. It is very important that the samples are analyzed immediately after collection, otherwise they must be stored in a refrigerator at 4°C, because samples may be degraded significantly during storage at room temperature between collection and analysis, resulting in low BOD values.

Apparatus used for BOD measurement were:

a. Incubation bottles with a ground-glass stoppers with a capacity of 250 to 300 ml. The bottles were washed and cleaned with detergent, rinsed thoroughly and dried before use.

b. Incubator which is thermostatically controlled at 20 + 1°C, excluding all lights to prevent the possibility of photosynthetic production of dissolved oxygen.

Reagents required per liter for the experiment are as follows:

KH_2PO_4.....8.5 g

K_2HPO_4.....21.5 g

$Na_2HPO_4 \cdot 7H_2O$.....33.4 g

NH_4Cl.....1.7 g

In this solution, pH must be maintained at 7.2 right after preparation without any adjustment, otherwise must be discarded and a new solution prepared.

Other reagents used as a deoxidation solution and nutrient for microorganisms are:

a. Deoxidation solution,

Sodium Sulfate solution.....0.25N

b. Nutrient Substances,

Magnesium Sulfate solution.....22.5 g/l

Calcium Chloride solution.....27.5 g/l

Ferric Chloride solution.....0.25 g/l

Acid and alkali solutions are also used for neutralization of the sample.

Seeding: It is necessary to have present, a population of microorganisms capable of oxidizing the biodegradable organic matter in the sample. Some samples such as untreated industrial wastewater and wastes with extreme pH values, need to be seeded. The preferred seed is effluent from a biological treatment system processing the waste. In this experiment, seed was provided from Livingston (NJ) Wastewater Treatment plant activated sludge.

Dilution water is prepared by addition of a necessary volume of water in a reservoir with enough capacity for one experiment. Then 1 ml each of buffer solution, nutrient solution and sodium sulfate are added into the reservoir and is saturated with oxygen by bubbling air, which takes almost 10 to 12 hours or more. Dissolved oxygen in the saturation state should be 8.6 mg/l. Once the diluted water is prepared, a proper fraction of samples containing 5%, 10% and 15% are pipetted in the BOD bottles. A small amount of seed 0.5 to 1 ml is added and the rest of the volume is filled with air-saturated diluted water. Overflowing the bottle takes out all the air bubbles from the solution, then the bottle is tightened with a ground

glass stopper. A check is made once or more to see if any air bubbles remain inside, then it is incubated at 20 $\pm$ 1°C.

Glucose-Glutamic check solution is prepared using 150 mg glucose and 150 mg glutamic acid. These substances are weighed and dried at 103°C in an oven for 1 hour, then are dissolved in 1 liter distilled water. Two percent of this solution is placed in a BOD bottle with seed and volume with diluted water to the top and incubated for 5 days. This solution is prepared for checking the biodegradation of organic compounds. If the BOD value of this solution is about 200 $\pm$ 37 mg/l then dilution water is good, otherwise it must be discarded and prepared again. Two blank solutions are also prepared, one with only dilution water and the other with dilution water plus seed with the same concentration as a sample. Dissolved oxygen of the samples are measured initially and every day for 5 days. In blanks and glucose-glutamic check solution, it is determined initially and after 5 days.

4.5.2. DISSOLVED OXYGEN UPTAKE (DOU)

The dissolved oxygen uptake was determined by using Method 213-A of the Standard Method for the Examination of Water and Wastewater [31]. An Orion Research Model 97-08 dissolved oxygen electrode was calibrated and inserted along

with a magnetic stirring bar into the BOD bottle, while the bottle was being placed on a magnetic stirrer plate. Dissolved oxygen readings were taken initially and every day. Dissolved oxygen was read on a digital ion analyzer (Orion Research Model 501). Results were analysed and computed in accordance with Method 507 of Standard Methods for the Examination of Water and Wastewater[31]. Dissolved oxygen uptake was also measured in short of time, 15 minutes. Samples were prepared with the same procedure as with the BOD preparation. The results were plotted against time. Parameter pH is important in dissolved oxygen measurement, and is maintained in the range of 5-8.

4.5.3 SUSPENDED SOLIDS

The suspended solids of stillage were determined by a modification of method 209-D of Standard Method for the Examination of Water and Wastewater [31]. Tared aluminum weighing dishes were used instead of ceramic crucibles. 30 ml of distilate bottoms were collected daily from a distillation flask. Three 10 ml aliquots of the liquid sample were pipetted into dessicated and preweighed aluminum dishes, (3 dishes per experiment). The samples were heated for 24 hours at 140^{o}C. After cooling in a dessicator for 5 minutes, the dishes were reweighed on an analytical balance

(Mettler type AE50) and the averaged solids concentration determined by the difference. Organic matter existing in this water was also measured by extraction but the substances were not identified.

4.6. RECYCLING OF STILLAGE

Recycling of stillage water was examined several times for hydrolysis of starch. Low quality pH from this water has been overcome by addition of neutralizers, prior to its consumption. Caustic substances such as calcium carbonate and sodium carbonate were used as neutralizers. The amount of calcium carbonate added to the distilled water for adjusting pH from 3.5-5.5 was 0.5% w/v. This amount, in case of using sodium carbonate was 0.1% w/v. Recycling was performed with total and one-third volume of stillage for estimation of better result and the most economic reuse of this process water.

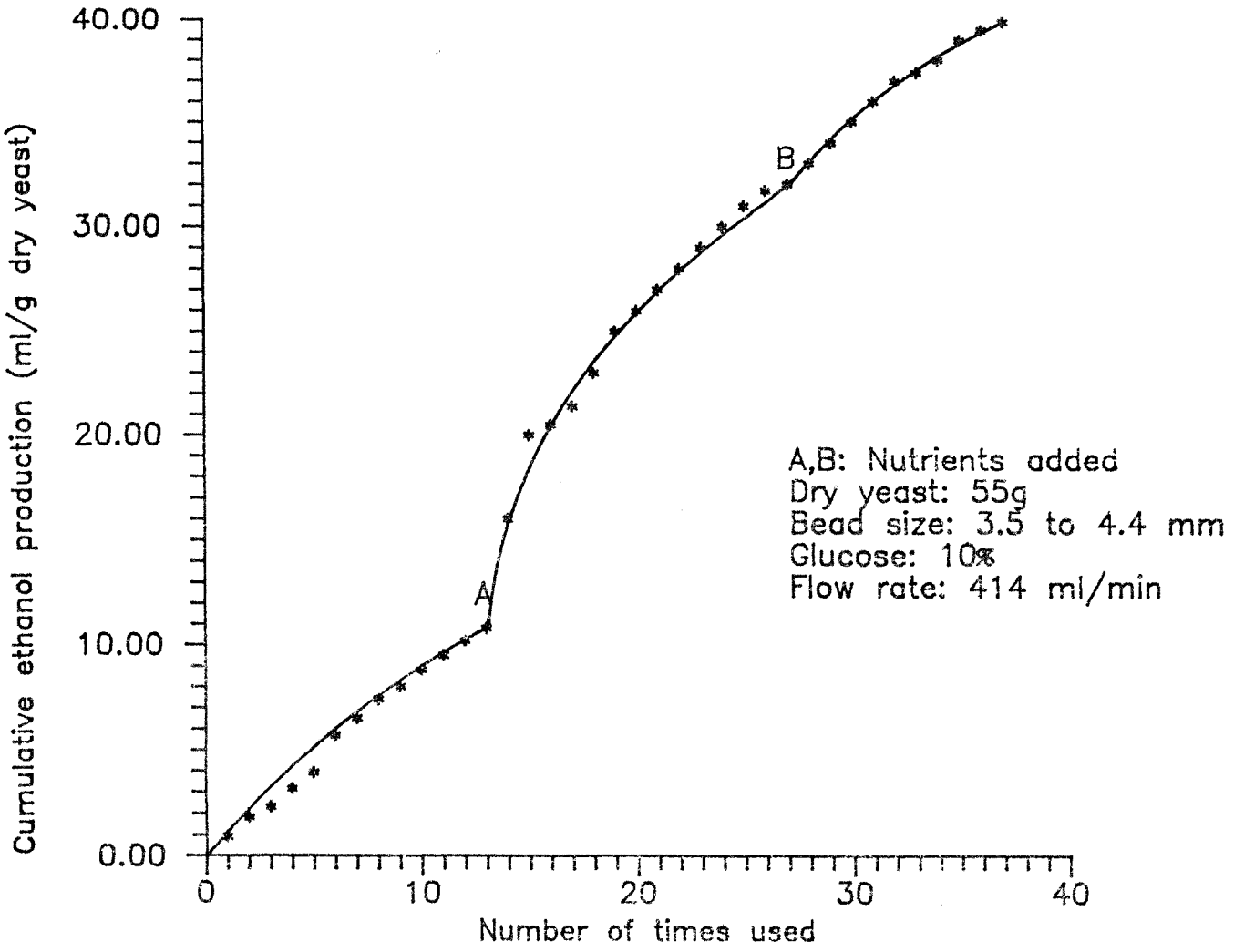
CHAPTER V

RESULTS AND DISCUSSIONS

5.1. ETHANOL PRODUCTION AND IMMOBILIZED YEAST CELLS

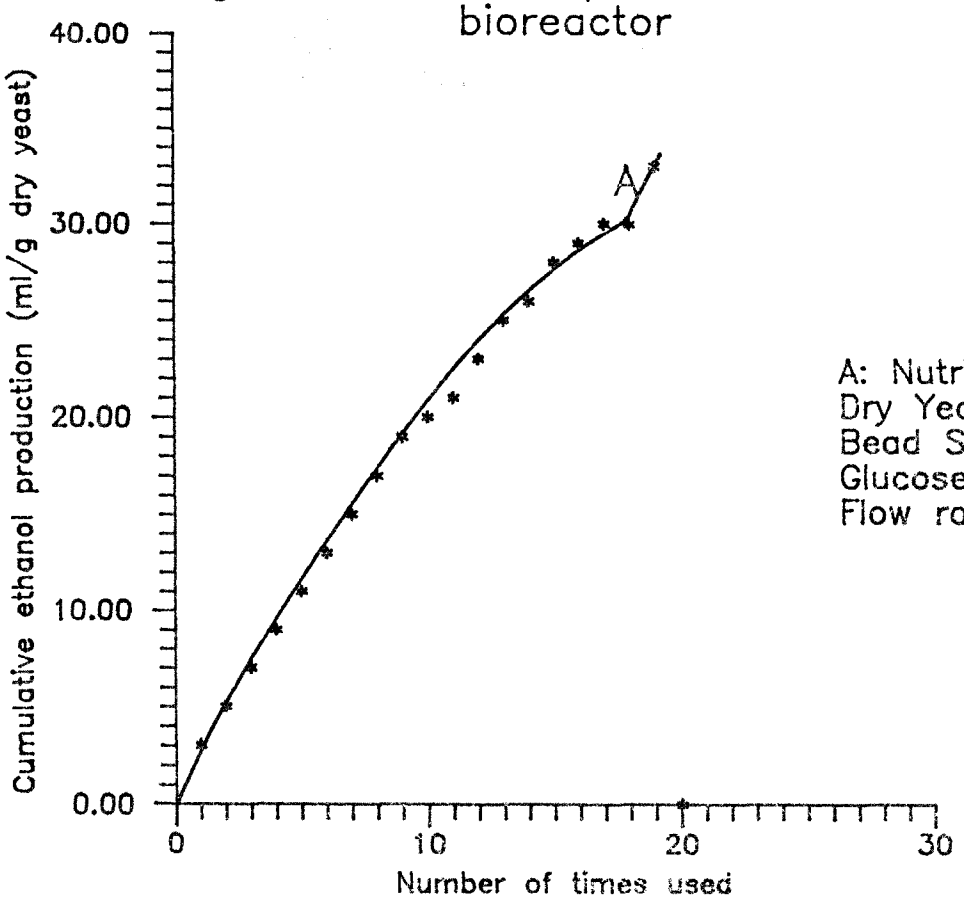
100 G active dry yeast cells were mixed with 7.5 g sodium alginate and dropwise extruded in 0.1 M calcium chloride solution for the formation of immobilized yeast beads. The beads were applied for fermentation of 10% w/v glucose in a recirculation reactor at 25 to 30^{o}C. The biomass loading of the reactor was 200 to 400 g wet immobilized beads, equivalent to 32-55 g dry yeast biomass. The size of the beads ranged between 3.0 to 4.4 mm in diameter. Reactor residence time was 6 to 8 hours and fermentation was performed in anaerobic conditions. The system continued to produce significant amounts of ethanol for over 37 times in run # 1, and 20 times in run # 2 (FIG. 3 and 4). Beads were still viable and active, in run 1. In run 2 beads were completely inactive and their oxygen consumption was nondetectable in use number 20. The reason can be either contamination with other microorganisms or inhibitory effect of nutrient. Storage of beads in 0.1 M calcium chloride solution after each use or 100-120 hours continuous fermentation helps alginate gel's strength but its effect on ethanol productivity needs more cautious

Figure 3. Ethanol production in recirculation bioreactor.



Production of ethanol in a recirculation reactor using the same batch of yeast for 37 times, with periodic addition of nutrients

Figure 4. Ethanol production in recirculation bioreactor



A: Nutrients added
Dry Yeast: 37g
Bead Size: 3 to 3.6 mm
Glucose: 10%
Flow rate: 660 ml/min

(i)Production of ethanol in a recirculation reactor using the same batch of yeast for 20 times, with periodic addition of nutrients.
(ii)No activity was observed when the beads were used on the twentieth time, probably due to contamination

experiment action. Analytical measurement of ethanol production in this reactor set up showed a concentration of 2-4% w/v and a rate of 0.054-0.002 l/g dry yeast. The cumulative ethanol production over a 260 hour period (37 days) and 160 hours (19 days) from CO_2 production considering the temperature at 27oc were calculated and plotted against time, Fig.3 and 4 respectively. Effect of nutrient on yeast beads activity and ethanol production is also indicated in the same figures at points A and B in Figure 3, and A in Figure 4.

Ethanol production studies were accomplished by run # 3. The run was divided into three continuous intervals, controlled by computer as mentioned in Chapter 4, and 15 single experiments, each conducted for 6-8 hours. Complete material balance data were not gathered because controlling the system with computer was the main purpose of this run, meanwhile viability of the beads was observed.

Studies showed [4], that the specific rate of ethanol production by the immobilized cells was much greater than for the suspended cells. Glucose was taken up by the immobilized cells at about twice the suspended cell rate, which is an unexpected result since the cells' surface area available to the immobilized cell for transport was probably lower.

Glucose absorption by alginate gel support was determined in this study. Results showed a very low amount of absorption of glucose by gel support.

In regards to contamination with the increased rate of glucose consumption and ethanol production in the immobilized cells, there was a decrease in their specific growth rate and ethanol production.

As mentioned before recirculation reactors have advantages in standard continuous industrial devices. So far, both the free cell recycle reactor and the immobilized cell reactor have been established. The immobilized cell reactor seems to have more advantages than the free cell reactor. While cell recycling requires additional equipment such as a settler or centrifuges, immobilized cell reactors represent a less costly and less complex solution.

5.2. EFFECT OF FLOW RATE

Experiments were run in varied flow rates of 660-330 ml/min. The ethanol productivity showed an increase by increasing the flow rate. The maximum ethanol obtained at 660 ml/min was 0.002 l/g dry yeast per fermentation in run # 2 and at 414 ml/min was 0.057 l/g dry yeast in run # 1. By maintaining constant flow rate, the rate of production would stay the same .

Flow rate has a dual effect on the system. At low rates, the system may be under the limitation of external mass transfer. On the other hand, at very high flow rates the beads' structure is endangered. Bead volume has an effect on flow rate. An increase in bead volume increases resistance to flow rate and also causes problems due to the prevention of CO_2 flow.

5.3. EFFECTS OF ENVIRONMENTAL CHARACTERISTICS ON ETHANOL PRODUCTION

5.3.1. Temperature: Temperature has an important influence on the growth of microorganisms including yeast and the rate of ethanol production. Temperature was maintained at 25 to 30^{o}C(room temperature) through all three runs. Low temperature showed none or very poor reaction, while high temperatures can have an influence on the chemical produced by microorganisms. Thus, shifts in temperature may vary the amount of pyruvate going into ethanol, organic acids and other alcohols.

Previous studies [3,5] showed that the immobilized system should be operated at lower temperatures, than with the free cell system, to minimize ethanol inhibition and maximize ethanol production.The ethanol inhibition effect will begin almost at 7 wt% ethanol [3].

5.3.2. pH: External pH is a very important factor for cellular growth. pH was maintained in the range of 4.5 to 5. However, analysis of samples of fermentation broth indicated that pH falls to 3-3.6 and remains the same in produced water after removal of ethanol. The optimal pH for ethanol production of free cell suspension was found to be 4.5. However, the immobilized system showed a broad optimum with most of the activity between pH of 3 to 7.5.

5.3.3. Nutrients and Minerals: Fermentation experiment were primarily conducted only with glucose that was extracted from cornmeal. A complete nutrient medium was added when bead cell activity and ethanol production were reduced. In run # 1 nutrient was added twice on day 17 and 27, points A & B in Fig. 3. Ethanol production indicated a significant difference before and after nutrient addition,(0.08-0.2 l/ h/g of dry yeast).

Several studies have been conducted for the effect of inorganic nutrient and ethanol production. In regards to the addition of NH_4^+ion as nitrogen source,[25] it was found that this element has marked effects on yeast, and is reflected by increased metabolic rates but no evidence of higher ethanol production was observed. Calcium ion was found to be the most inhibitory component tested, and it is also present at substntial concentrations in carbohydrate

sources [25]. On the other hand this element is found to be very effective for hydrolysis of starch. Although phosphate ions are essential to maintain microbes, they should be excluded or reduced from the nutrient medium because they have a deteriorating effect on the calcium gel.

5.4. CELL GROWTH AND DENSITY

Cell growth and density were studied by determination of protein concentration, using a total protein measurement prccedure. Protein concentration of beads which were measured often during repeated fermentation increased from 20.83 mg/g wet to 60.91 mg/g wet, (Table 2). A microscopic examination of immobilized cell beads indicated the division of some of the cells during the fermentation process at suitable conditions of pH, temperature, and residence time. This was confirmed by taking samples of the solution and measuring the absorbance at 600 nanometers.

The number of cells were counted microscopically after the separation of alginate gel from cells and preparation of a series of dilutions on a lined chamber. The number of cells showed an increase due to repeated fermentation process. This number changed from 13 x 10^6/l to 14 x 10^6/l. The cells were also larger in appearance.

Table 2 - Protein Content of Beads During Continuous Fermentation

Number of Use	Protein (mg/g wet)
0 (initial)	20.83
7	29.28
14	30.00
19	35.45
24	37.50
37	60.91

5.5. VIABILITY AND OXYGEN CONSUMPTION OF BEADS

The viability of beads was studied as beads were under a repeated fermentation experiment. Viability was measured using a microassay reactor by the rate of oxygen consumption. The rate of oxygen uptake by the beads is indicated in Figure 6. Immobilized bead cells initially started to consume oxygen at rate of 86.42 nmol/min. As they were used in the fermentation process, their oxygen consumption increased to about 180.35 nmol/min. Performance of cell growth can be reasons for this increase. The subsequent lowering of the rate of oxygen consumption may be due to the beads adaptation to anaerobic conditions of fermentation. When their medium condition changes to an aerobic state, the ability of consuming oxygen reduces but it doesn't stop as long as they can produce alcohol.

5.6. ASSESSMENT OF POLLUTION

5.6.1. BIOLOGICAL OXYGEN DEMAND (BOD)

Determination of BOD was mainly performed in produced water and filter cake. For each analysis three different concentrations of samples were prepared, 5%, 10%, and 15% of the volume of the BOD bottle. Samples were incubated at $20\pm1^{o}c$ for 5 days without exposure to the light. Dissolved oxygen was measured initially and after 5

Figure 5. Viability and Oxygen consumption of beads.

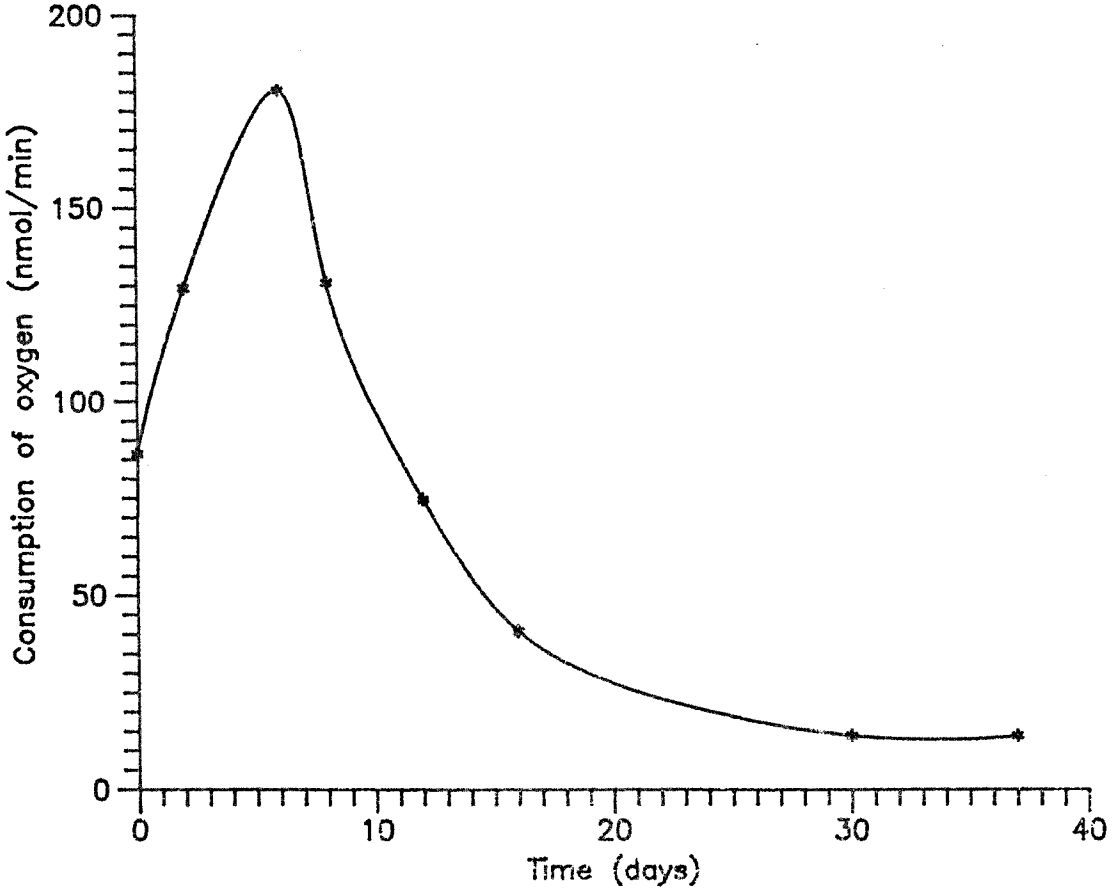
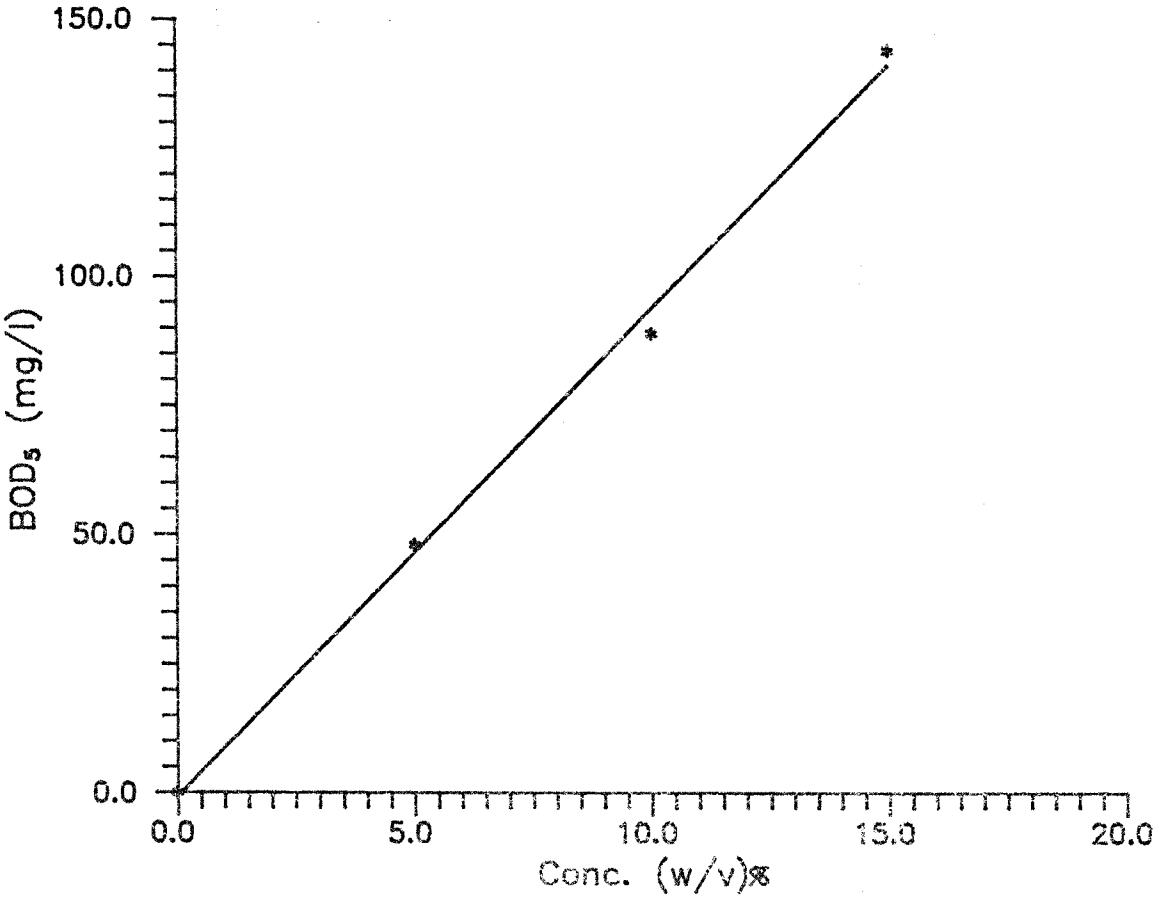


Figure shows oxygen consumption of a batch of beads over a period of 37 days of its repeated use.

days incubation for calculation of BOD_5. As seen in Fig. 6 and 7 the higher the concentration of substance, the greater the BOD level. It means there is a linear relationship between the concentration of organic substances content of the samples and the level of BOD. In filter cake the average BOD was found to be in the range of 360 ± 10 mg/l, and in recycled water was 115-360 mg/l. Therefore, treatment of this water is required before its discharge in the waste stream. Filter cake contains a substantial amount of protein (nitrogen containing compounds) which is able to consume oxygen very fast. Variation in BOD level of filter cake can be affected by the amount of its protein content. The fluctuation in BOD level of recycled water depends on the removal of ethanol . Ethanol is found to have a high level of BOD. The average BOD level of a fermentation liquid in which ethanol is not yet removed was about 1350 mg/l. This liquid contains at least 1-3% w/v ethanol. In ethanol technology, ethanol as a final fermentation product is not wasted. Therefore one is normally not worried about existence of ethanol in water because it is ultimately stripped out. Fig.6 shows a linear relationship between BOD and the organic substances such as protein and ethyl alcohol.

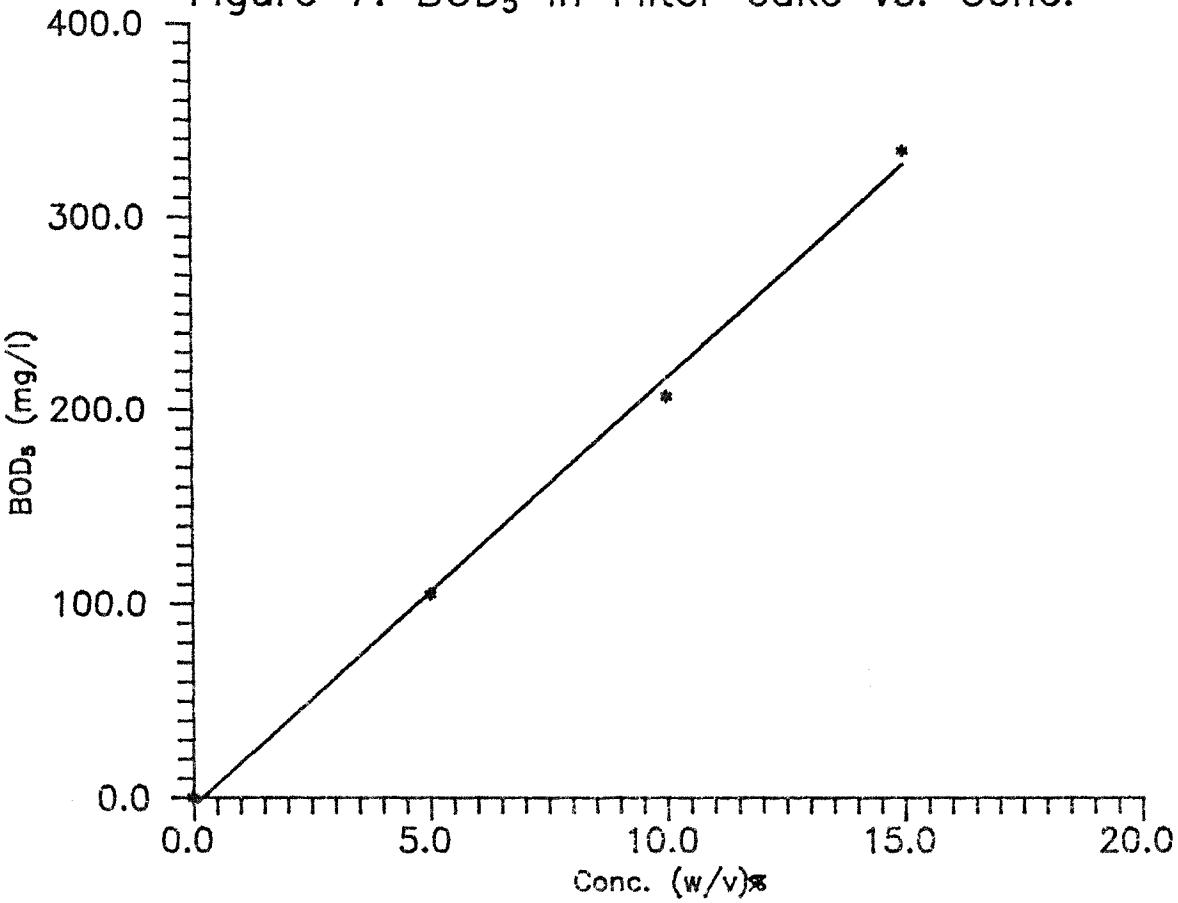
It was found that the average BOD load due to the continuous recycling of process water decreases. This is an advantage of recycling.

Figure 6. BOD_5 of Stillage Vs. Conc.



There is a linear relationship between stillage concentration and BOD_5.

Figure 7. BOD_5 in Filter cake vs. Conc.



There is a linear relationship between concentration and BOD_5 level.

The BOD of a wastewater containing certain amounts of the nonbiodegradable substances such as DDT, is essentially zero, while in water containing the same amount of sugar, it is very high. Raw domestic sewage has a BOD of about 200 mg/l. Some food processing wastes have BODs of over 100,000. Drinking water BOD should be zero; lakes should be below 2 mg/l. However, a high BOD indicates the abundance of organic substrates, that can be used by microorganisms aerobically. When the BOD is high, however, microorganisms often use much of the available oxygen for the degradation of organic matter.

The dissolved oxygen uptake rate of process water in a short period of time (15 min.) was measured. 5%, 10% and 15% of the sample in BOD bottle with capacity of 300 ml were prepared. Microorganisms were added for evaluation of biological activity. This measurement was done initially and after 5 days incubation. The D.O. uptake by microorganisms was very slow, the amount of almost 2 mg/l/day was consumed. Microorganisms indicated almost the same rate of activity in initially prepared samples and after five days incubation.

The rate constant of BOD was calculated mathematically, which shows the rate of 0.1 for stillage and filter cake [Appendix B].

5.6.2. SUSPENDED SOLIDS

Process water (stillage) was analyzed for determination of suspended solids. The solids content was measured by the evaporation of water and weighing the precipitated part which was obtained around $1.2x10^{-3}$% w/v.

Organic compounds as measured by extraction using methylene chloride as solvent, was about $3x10^{-3}$% w/v. Solids also consist of a small amount of alginate gel while separated from beads during fermentation process and also inorganic substances from addition of nutrient and caustic reagents added for adjusting pH. This concentration, however, is not very high. Organic compounds consist of acetic acid, very low amounts of unremoved ethanol and possibly some butyric compounds. The importance of solids in BOD analysis, is that the amount of oxygen consumed by bacteria in the presence of organic or inorganic compounds depends on the concentration of the solids content of wastewater. The higher the solids content, the higher the BOD level.

5.6.3. EFFECT OF RECYCLING ON POLLUTION

Process water or stillage as a wastewater product in ethanol technology was recycled for the reduction of volume of water discharged in the waste stream, lowering the costs of treatment and the low cost of ethanol production operation. Theoretically, in metabolic conversion of sugar

to ethanol, the end product of sugar fermentation by yeast cells in an anaerobic condition is ethanol and a very low concentration of acetic acid or pyruvate.

Ethanol is separated from fermentation broth at the end of fermentation process as efficiently as possible. Concentration of the other compounds in the water are not very high. On the other hand, their presence is not hazardous for further ethanol production process. Therefore this water can be reused in ethanol production without causing any major problem as it was tried in this study.

In this experiment recycled water was used for hydrolysis of starch to produce sugar for fermentation. For five runs, the total volume of water required for hydrolysis process was provided only from recycled water without addition of any make up water. In other trials, one-third of water required for hydrolysis plus two-thirds of make up water was used. In all trials, hydrolysis of starch was completed successfully after adjusting pH for enzyme activity. There were no signs of any major problems in starch hydrolysis and fermentation.

Recycling of this water not only has many advantages including reduction in the volume of discharged water, lower consumption of processed water, saving in energy, nutrient and enzyme. Above the all, reduction in production cost and also pollution treatment cost are the advantages.

5.6.4. EFFECT OF CIRCULATION ON ETHANOL PRODUCTION COST

A. Pretreatment Cost: Operating expenses also vary with the processing required by different feedstocks. Operating expenses are not only related to the equipment required, but can also be affected by such things as the requirement of energy or the quantity of enzyme required. Recycling of distillage can save quite a lot in energy and cost of make up water, as well as the cost of pollution treatment.

B. Relative Cost of Yeast: Using the same amount of yeast entrapped by alginate gel support repeatedly in fermentation has many advantages including cost of the yeast cell.In this study, the average amount of alcohol produced was 0.054-0.002 l/g dry yeast per fermentation. While cost of yeast remains the same, ethanol continues to be produced as long as yeast are alive. Fig. 8 shows a relative cost of yeast in after 37 fermentations. Repeated use of the same yeast beads not only has effect on the cost of the yeast, but it has also an effect on the materials used for immobilization.

C. Relative Cost of pollution: Rrecycling of process water for ethanol production processes has many advantages. Reduction of pollution and the cost of treatment is one of the major advantages of reusing of the produced water. Fig. 9, shows a relative cost effect on the water pollution in bio-ethanol technology. As seen in the figure recycling of

process water in a repeated fermentation process for 37 times reduces its relative treatment cost substantially.

Figure 8. Effect of recirculation on the relative cost of Yeast

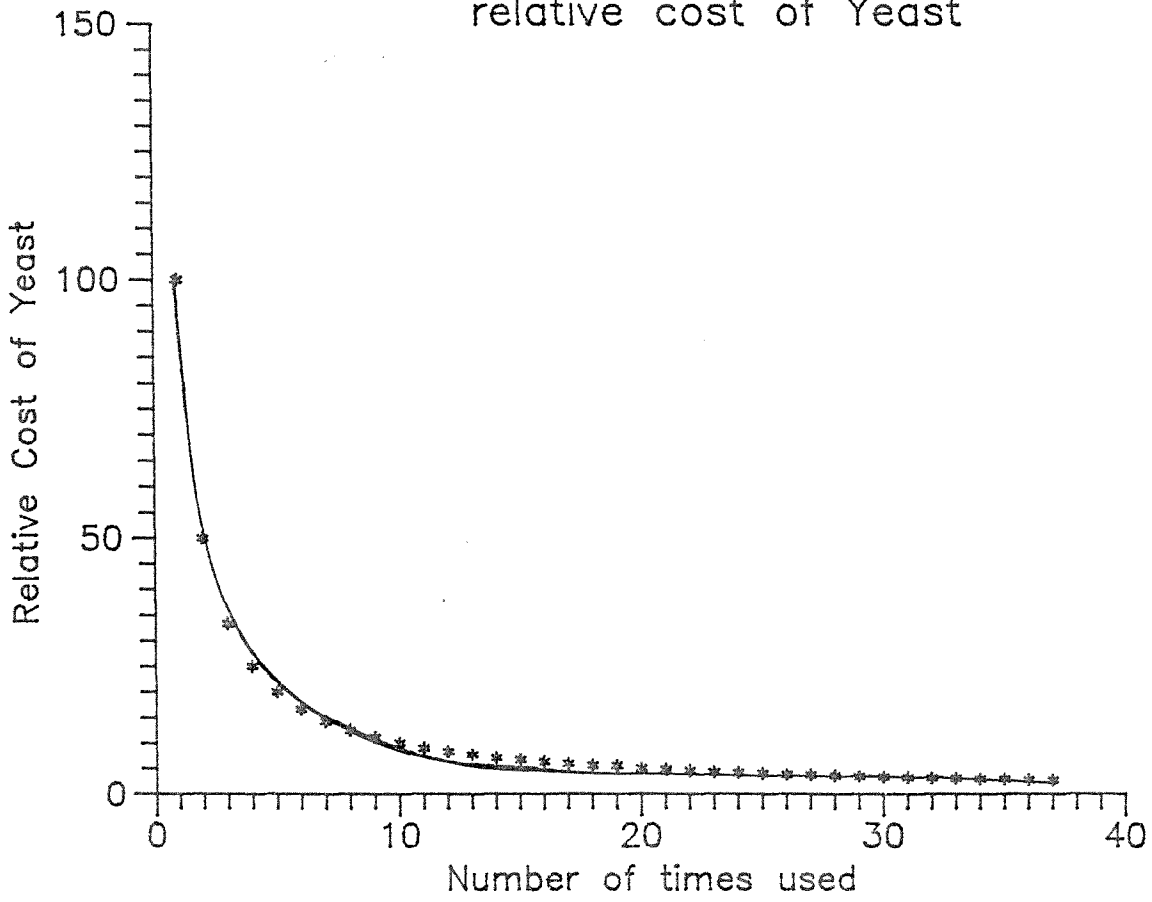


Figure indicates relative reduction in cost of yeast by reusing it 37 times

Figure 9. Effect of recirculation on relative cost of pollution

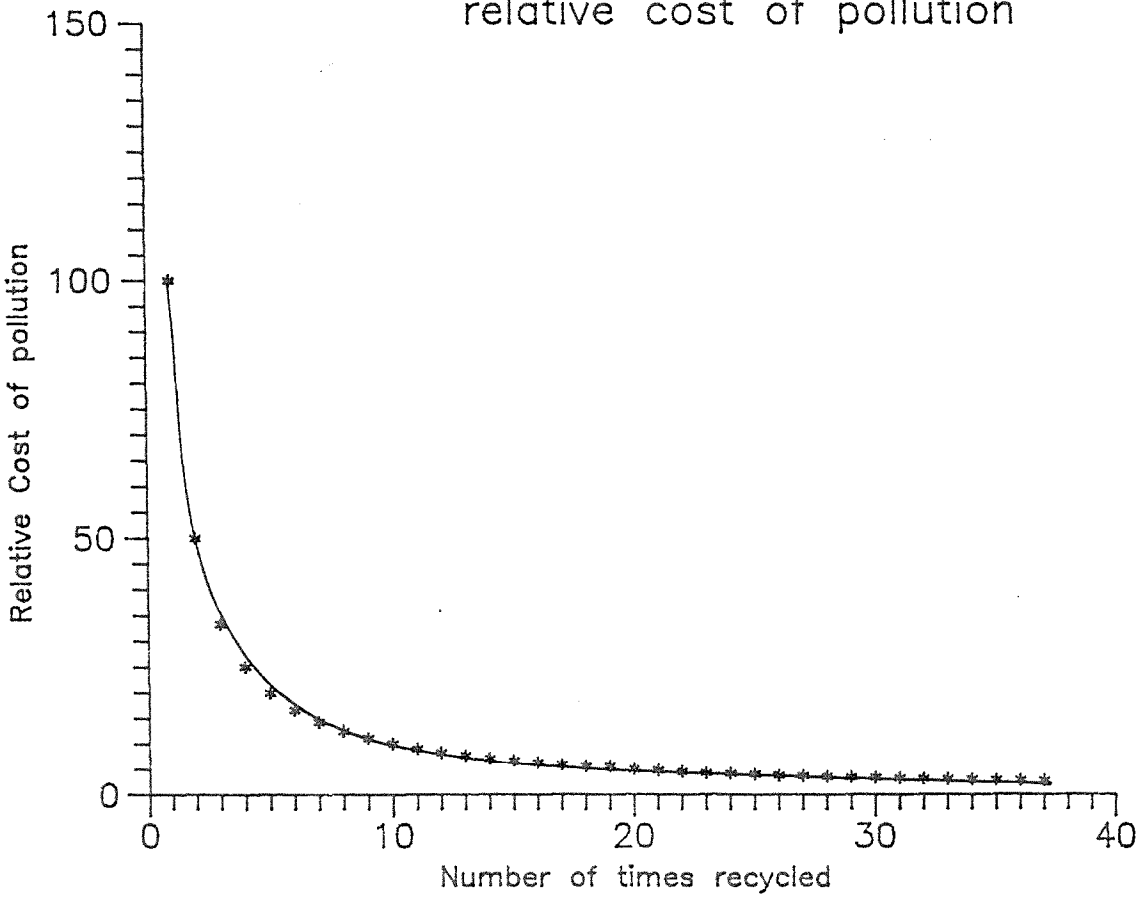


Figure indicates relative reduction in cost of pollution by reusing the stillage 37 times

CHAPTER VI

FILTER CAKE AS HIGH-PROTEIN SUPPLEMENTARY FEED

Generally, in ethanol technology the choice of feedstock (corn) has a significant effect on process operation. Fermentable solids can be obtained from several sources. The extremes in choice extend from biomass production devoted to ethanol fermentation, to pretreatment of waste streams. Some feedstocks will affect capital and operating costs by their waste stream treatment requirements. These feedstocks can produce high biologcal oxygen demand and high salt streams with little value such as animal feed. Corn is one of the feedstocks, which is a valuable animal feed and also very low in BOD [2].

Today, in the United States most fermentation ethanol is made from corn. Corn is roughly 80% starch, which is a polymer of glucose. Enzymatic hydrolysis of starch produces individual sugar molecules that are then fermented to produce ethanol. The residue of saccharification slurry or filter cake contains high amounts of protein that can be used as a high-protein food product. Total protein content of this cake was determined by using a total protein measurement procedure from Sigma Chemical Company.

By comparison, composition of cornmeal [34,35] is as shown in Table 3. By considering the nutritional requirements of beef and dairy cattle, filter cake can be a valuable supplementary feed. The nutritional requirements of cattle vary with their body size, age, activity of the animal and fat content found in the milk. For example, a growing dairy Heifer and bull calves fed only milk and weighing 25 kg, with small frame size, one week old and daily recomended weight gain 300 g, need 111 g total crude protein; while growing dairy Heifer with 100 Kg body weight, small frame size, 26 weeks of age, 400 g daily weight gain, require 336 g crude protein.

Generally, protein requirements of dairy cattle is vary between 111-243 g daily. In lactating and pregnant cows, this amount is much higher and ranges between 300-1136 g based on a daily requirement.

PROTEIN AND ITS IMPORTANCE

Protein is required to furnish the animal with amino acids, which are necessary for various essential synthetic processes in the body. Amino acids are the building units of all cells and tissues in the body, including the blood, skeleton, vital organs, brain, muscles and skin. All protein secretions in the body including enzymes, hormones, mucin, and milk required a specific assortment of amino

Table 3 - Composition of Cornmeal

Starch	78.4%
Water	12.0%
Protein	7.8%
Fats	1.2%
Ash, Fibers	0.6%

acids. In rumminating cattle, the amino acids required may be obtained from dietry protein and some nonprotein nitrogen compounds such as urea. Protein is specially important for the lactating cow, because milk solids contain about 27 percent protein and cow secreting 30 kg of milk daily makes about 1 kg of a new protein for the milk. This amount is similar to that which might be produced by a 6-7 kg gain in body weight.

Comparison of protein content of corn [35], with protein content of the filter cake produced from the ethanol production plant, with dairy and beef cattle's daily protein requirements, Table.4 indicates that filter cake in its dried state; the form which is considered to be supplied as animal feed contains over 28-33% w/w protein. Therefore, it can be said that from an alcohol plant with a working capacity of 6.66 kg feedstock (specifically cornmeal) per day and the production of 4.25 l ethanol, about 9 kg wet and 4 kg dry high protein filter cake is obtained. This amount, referring to the above mentioned information, can provide protein requirements of a growing dairy Heifer, with 100 kg body weight, small size, 26 weeks of age, 400 g daily gain, requiring 336 g crude protein per day. For 12 days if fed as a total protein requirement and for 36 days if one-third is added to its regular daily feed as a supplement.

Table 4 - Composition of Filter Cake in Wet and Dry State

	Dry % w/w	Wet% w/w
Protein	28.4-33	15.0
Starch	31.0	16.3
Water	-----	47.4
Glucose	36.6	19.2
Ash	1.9	0.99
Fiber	2.1	1.10

CHAPTER VII

CONCLUSIONS AND SUGGESTIONS

7.1 CONCLUSIONS

After many trials, a continuous recirculation reactor applying immobilized yeast cells was established and operated. Experiments were conducted based upon previous studies [3]. Parameters that have significant influence on the activity of immobilized yeast, such as pH, temperature, concentration of substrates and nutrients were under control during reactor operation. Parameters such as flow rate, mass transfer resistance and bead size were reinvestigated in ethanol production, based on previous studies [3].

Size of beads has an important role in ethanol productivity. Smaller diameter beads, responded to a higher ethanol production than larger diameter beads. The flow rate significantly influences bead performance. Higher flow rates result in a higher ethanol production in the immobilized cell reactor. However, at lower rates the overall reaction rate is much lower than the reaction rate of the free cell suspension, due to the mass transfer limitation, while at higher flow rates, larger beads have a larger intraparticle diffusion requirement, which leads to a smaller overall reaction rate.

Bioreactor loading with beads must be taken under consideration. An excessive amount of beads in the reactor results in the formation of a dense packing that could lead to fluid channeling and also block the release of CO_2 gas, causing leakage and operational problems.

In practice, ten percent glucose medium (180 g/1.8 l) has been used for each experiment. In 37 fermentation experiments a total amount of 6.66 Kg glucose was used. This glucose was extracted from conversion of 9.8 Kg starch obtained from 12.5 Kg of cornmeal, assuming that 77.8% of cornmeal is starch. Experimentally, an average amount of 0.054-0.002 l/g dry yeast alcohol equivalent to 95% alcohol was produced in entire fermentation experiment. Volume of ethanol was calculated based on the density of alcohol as 95% alcohol. Analytical measurement indicated a 2-4% concentration of ethanol. Theoretically, the conversion of 6.66 Kg glucose would produce 4.25 l ethanol, while in practice 3.37 l ethanol at 95% concentration was obtained. As can be calculated, there is a difference of 0.88 l or 20% in ethanol produced theoretically and experimentally. This difference can be due to the contamination of beads with other microorganisms or operational error.

Generally, it can be said that fermentation reaction was more successful than glucose extraction. Based on the assumption, 90% of starch equivalent to 79.17% must be

converted to the glucose, while our calculation indicates 61.6% production of glucose. Of course the amount of 3-5% glucose remained in the filter cake should be taken under consideration.

In this study, immobilized yeast showed a fair amount of ethanol productivity and growth only in glucose media, however, the addition of nutrient indicated higher ethanol productivity even though it was added after several fermentations.

Water produced after the distillation of alcohol (stillage), was analyzed for both recycling and BOD determination. Recycling of this water in the starch hydrolysis process after neutralization with caustic compounds for pH levels, was successful. However, although dissolved oxygen uptake was slowly performed in stillage, but a D.O.uptake of 2 mg/l per day by microorganisms was observed. In various industrial treatment plants, dissolved oxygen is maintained at different ranges, but generally, in conventional waste treatment aeration systems, dissolved oxygen is maintained in the 1-3 mg/l range. A varied level of BOD between 115-360 mg/l was obtained in the stillage. Any waste water within the range of this BOD value should be treated before it is discharged into waste stream.
On the other hand, reuses of this water after adjusting for low pH value was successful.

BOD was also measured in filter cake. The BOD of filter cake was determined in the level of 360±10 mg/l, which can be a cause of pollution in bio-ethanol technology. However, filter cake contains a valuable amount of protein (28-33% in dry state) that can be used for animal as high-protein supplementary feed. This is an advantage of using corn as substrate for ethanol production as well as its low BOD value in comparison with other biomass used in ethanol technology. Consumption of filter cake as high-protein feed can also help the existing problem about injection of hormones to the dairy and beef cattle for increasing the growth and productivity.

7.2. SUGGESTIONS

In the present study, although ethanol production using a recirculation reactor with immobilized yeast was conducted well, and ethanol production was relatively high, but yet more improvement has to be made. Any contamination either in beads or in reactor must be avoided. Environmental factors should be controlled closely for the better ethanol productivity and beads cells activity. Concentration of glucose for fermentation can be increased, but more than 20% is not suggested. Although beads were changed in size and shape during glucose utilization, the alginate gel beads productivity still is unclear, in which further studies are

required. Building a reactor with a rotary beads bioreactor seems to prevent mass transfer resistance and the dense packing.

Primary studies of pollution evaluation has been conducted in water after the distillation of alcohol and filter cake, in which more investigations are required. Designing a recirculation reactor for the treatment of produced water and determination of biological activity in this water could be more effective tool than performing a tedious, time-consuming BOD analysis.

Finally, since the microprocessing plant will allow external monitoring for all ethanol processing conditions, a computer for such a plant will be unique and, like the plant. Miniature slave microprocessors can control and monitor the plant, communicating externally with a microprocessor located elsewhere. A master processor located centrally, could monitor the functions of several remote microprocessors [37].

APPENDIX A

Calculation of BOD

When dilution water is not seeded:

$$\text{BOD, mg/l} = \frac{D_1 - D_2}{P}$$

When dilution water is seeded:

$$\text{BOD, mg/L} = \frac{(D_1 - D_2) - (B_1 - B_2)\, f}{P}$$

Where:

D_1 = D.O. of diluted sample immediately after preparation, mg/l

= 8.1 mg/l

D_2 = DO of diluted sample after 5 days incubation at $20^{o}C$ mg/l

= 0.2 mg/l

P = Decimal volumetric fraction of sample used

= 0.05

B_1 = D.O. of seed control before incubation, mg/l

= 7.7 mg/l

B_2 = D.O. of seed control after incubation, mg/l

= 7.00 mg/l

f = Ratio of seed in sample to seed in control

= (% seed in D_1)/(% seed in D_2)

BOD (mg/l) = 144

APPENDIX B

Calculation of BOD rate constant:

Rate constant (K) of BOD is measured as follows:

Y = BOD

$$Y = 1 - (2.3\ K_t)\ [\ (1 + 2.3/6)\ K_t\ ]^{-3} \quad \text{-----(1)}$$

Rearrangement of equation (1) may be transformed as follows,

$$(t/y)^{1/3} = \frac{1}{(2.3\ K\ L)^{1/2}} + \frac{(2.3\ K)^{2/3} t}{6\ L^{1/3}} \quad \text{-----(2)}$$

Equation is linear in $(t/y)^{1/3}$ and t as shown Fig 10.

The intercept and slope of equation (2) are obtained by,

$$A = (1/2.3\ K\ L)^{1/2} \quad \text{------(3)}$$

and $$B = (2.3\ K)^{2/3}/(6L^{1/3}) \quad \text{------(4)}$$

(i) From experimental results, Y and t calculate the value of $(t/Y)^{1/3}$ for each day.

(ii) Plot $(t/Y)^{1/3}$ verses t on a graph paper and draw a best fit line as shown in Fig. 10, 11.

(iii) From the plot measure the intercept A and the slope B.

(iv) Finally, calculate K and L from the equations:

$$K = (2.61\ B)/A \quad \text{------(5)}$$

$$L = 1/(2.61\ K\ A^2) \quad \text{------(6)}$$

K = BOD rate constant

L = BOD rate constant

In a rearranged and simple form,

$$K_{10} = 2.6(m/b)$$

where

K_{10} = Rate constant and

m/b = slope

Figure 10. Linearized form of the BOD Equation for stillage

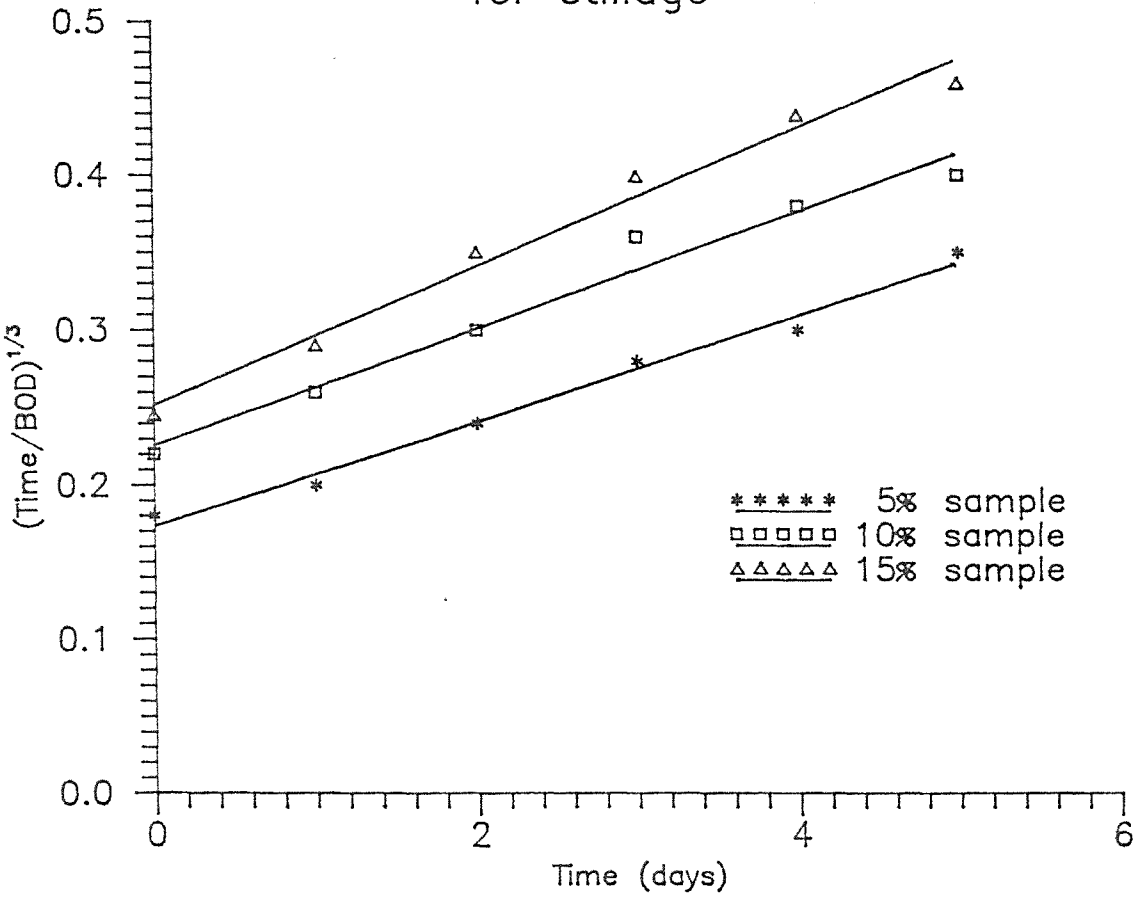
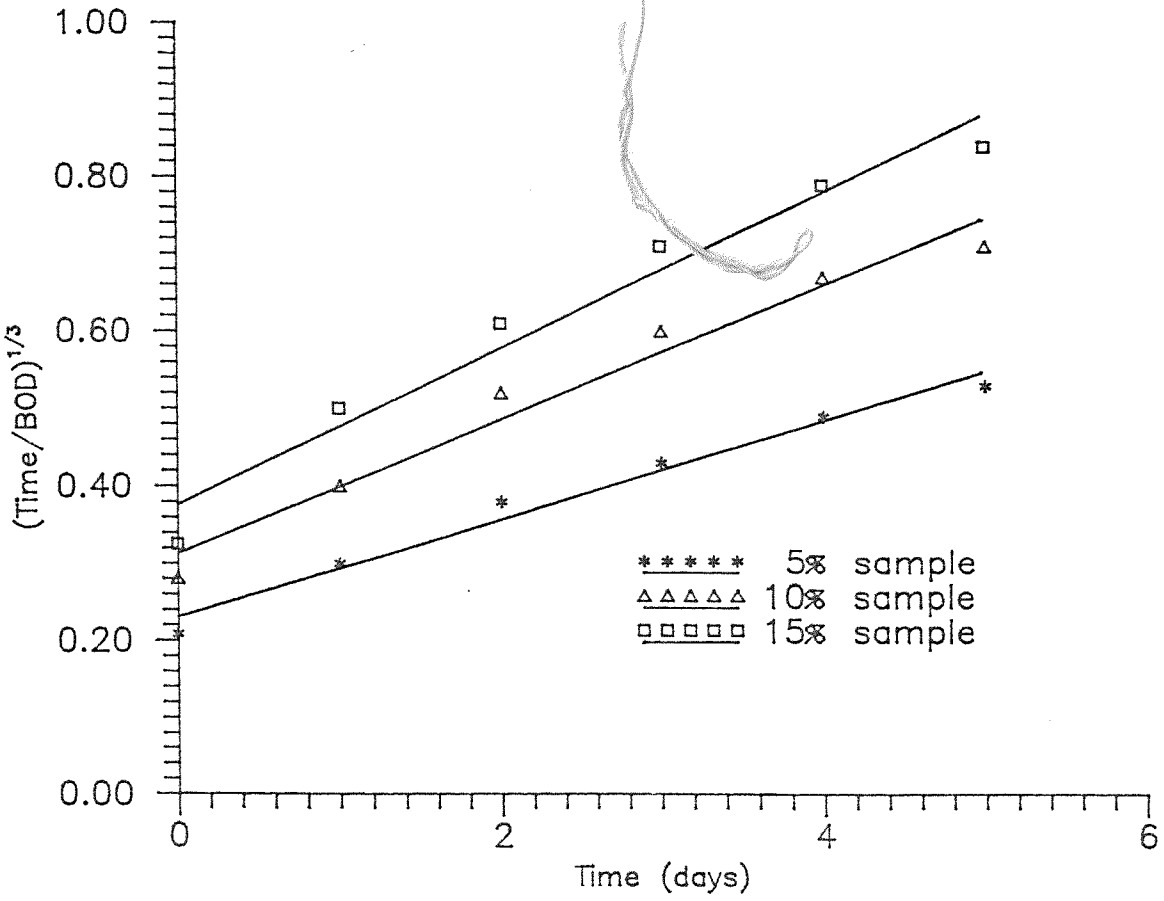


Figure 11. Linearized form of the BOD Equation (filter cake)



BOD rate is shown to increase with increasing ethanol concentration.

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