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Thesis Title:	Biomass conversion of Solid-waste (newsprint) into a clean Fuel (ethanol) by Enzymic hydrolysis and Fermentation
Name of Candidate:	Nimesh V. Gandhi M.S. in Environmental Science, December 1991
Thesis Directed by:	Dr. Sam S. Sofer, Professor Department of Chemical Engineering, Chemistry and Environmental Science.

ABSTRACT

A feasibility study has been performed with two bioprocesses for conversion of newsprint waste to an environmentally safe fuel (ethyl alcohol) and carbon dioxide.

The following areas were investigated to accomplish this task: one hour pretreatment of newsprint by attrition, saccharification of paper slurry to glucose by cellulase and cellobiase, effects of pH, temperature and concentration of enzymes on conversion of paper to sugar, fermentation of glucose to ethanol and carbon dioxide by immobilized yeast, and simultaneous saccharification and fermentation of paper to ethanol and carbon dioxide using enzymes and immobilized yeast.

Results indicate that the enzymic hydrolysis of newsprint to sugar and fermentation of sugar to alcohol by immobilized yeast is not only an efficient means to recover energy but also to treat the solid waste and thus help preserve the environment. However, a simultaneous saccharification and fermentation was found to be less favorable to produce the desired amount of glucose and ethanol.

BIOMASS CONVERSION OF SOLID-WASTE (NEWSPRINT) INTO A CLEAN FUEL (ETHANOL) BY ENZYMIC HYDROLYSIS AND FERMENTATION

By

NIMESH V. GANDHI

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

December 1991

Approval Sheet

Thesis Title: Biomass conversion of Solid-waste (newsprint) into a clean Fuel (ethanol) by Enzymic hydrolysis and Fermentation

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CHAPTER I

INTRODUCTION

1.1 ALTERNATE ENERGY RESOURCES:

Recovery of useful products and energy from refuse and agricultural wastes as a way to relieve their disposal and minimize the pollution problems has received intense national and international attention. The bioconversion of cellulosic wastes such as paper to ethanol for blending into gasoline to decrease U.S. dependence on imported crude oil and also to reduce uncontrollable automobile exhaust emissions has received increasing interest.

The microbial conversion of agricultural substrates such as corn-starch, wheat-straw, fruits and vegetables to sugar and alcohol is an ancient practice, currently involving microbiology, chemistry, and chemical engineering. Since much of the agricultural waste material is composed of cellulose, some attention has been focused on the hydrolysis of cellulose to glucose by enzymes. Glucose is commonly used by yeast or other microorganisms as the substrate for the production of ethanol and carbon dioxide.

The enzymic hydrolysis of waste paper, by cellulases, to glucose and subsequent fermentation of glucose to ethanol and carbon dioxide by yeast has been investigated by various

researchers. In this process, the hydrolysis of paper to sugar is recognized as the key step responsible for the technical feasibility of the overall process. Additionally and importantly, the utilization of this technique helps eliminate the problems associated with treatment and disposal of hazardous solvents generated from the pretreatment of waste paper prior to recycling, thus providing a strong alternative technology for recycling and environmental protection.

1.2 BIOCONVERSION OF NEWSPRINT TO SUGAR AND ETHANOL:

Enzymic hydrolysis of waste paper to sugar (glucose) and fermentation of glucose to ethanol and carbon dioxide have attracted many researchers to further investigate the technical feasibility of this process and its economic viability for implementation on commercial scale. The important factors associated with the entire process can be inexpensive, and there is continuous availability of raw materials like waste paper, enzymes, and yeast.

Biochemical conversion of waste paper, particularly newsprint, to sugar by cellulases has been studied by various researchers [3,4,5,6] primarily due to free and large scale availability of newsprint as a substrate. Lee and Jones [3]

found that simultaneous milling and hydrolysis of newsprint by cellulase in an attrition bioreactor (ABR) under specified conditions such as temperature, pH, enzymes concentration, milling media height, stirring speed, and in the absence of air-liquid interface was feasible. The absence of air-liquid interface during enzymic hydrolysis significantly reduced enzyme deactivation and hence may be an effective means to convert newsprint to sugar.

Deeble and Lee [4] report that enzyme deactivation increases with increasing power input in an ABR. In a similar study, Furcht and Silla [5] conclude that the rate of conversion obtained in a ball mill reactor (BMR) is less than that obtained in an ABR. However, the BMR required less energy than the ABR to reach the same extent of conversion. In an interesting study, Grethlein [6] has compared the process economics of making glucose from cellulose by acid (dilute H_2SO_4) hydrolysis on a plant design basis with that of a published design using enzymic hydrolysis. He reports that the enzymic hydrolysis process requires two times more fixed capital and it produces about 100 tons/day less glucose than the acid hydrolysis process. However, higher costs of energy and maintenance are involved in the acid hydrolysis process due to higher temperature and pressure requirements during the reaction. Also, since the acid hydrolysis produces byproducts, the purity of the glucose solutions obtained from

the acid hydrolysis process is not good when compared to that obtained from the enzyme hydrolysis process.

While much has been written about the hydrolysis of cellulose, and on fermentation of sugars, there is little information about a combined process which might be designed for bioconversion of waste paper to ethanol. The purpose of this study is to combine enzyme and whole-cell technology for conversion of waste paper into a clean fuel, with the long range intent of reducing overall costs, and especially enzyme cost.

Biomass conversion processes involving fuel manufacture have been an important research effort at the NJIT Biotechnology Laboratory in Newark, NJ. The overall objective has been to develop integrated bioconversion units. The advances made heretofore have been in the individual optimization of the enzymic systems, and of the fermentation system. We feel that only a combined enzyme/yeast/enzyme production plant has the potential of becoming economically feasible in the future. This thesis lays the experimental groundwork required for combining the individual units.

In this study, bioconversion of waste newsprint to sugar by enzymic hydrolysis using cellulase and cellobiase, and subsequent fermentation of glucose to ethanol and carbon dioxide by immobilized yeast were investigated as a basis for future work to optimize process conditions and to improve the

economics for this technology. The effects of temperature, pH and concentration of enzymes on conversion of cellulose to glucose were studied. The enzymic hydrolysis of paper to glucose was found to be particularly efficient when a mixed enzymic system consisting of cellulase and cellobiase was used. In the saccharification step, the presence of an air-liquid interface had adverse effects on glucose yields. The fermentation of glucose to ethanol and carbon dioxide, obtained from enzymic hydrolysis of cellulose, was achieved by conventional batch fermentation process using immobilized yeast entrapped within a calcium alginate gel matrix.

This research work describes a three step process for converting newsprint and waste paper into bioethanol and carbon dioxide using enzymes and yeast. The three step process includes: one hour pretreatment of paper by attrition, enzymic hydrolysis of paper to sugar, and fermentation of sugar to ethanol and carbon dioxide by immobilized yeast. Its application would result in the development of a renewable energy source from waste paper in the form of alcohol. It will also help in conserving natural resources, and reducing environmental impact. In addition, a combined hydrolysis and fermentation process was tested in a batch reactor to study a potential reactor configuration for large scale production of ethyl alcohol.

1.3 APPLICATION OF ENZYMES IN CELLULOSIC WASTE CONVERSION TO FUELS:

Production of ethanol from lignocellulosic wastes is a subject of intensive research because ethanol can be either blended with gasoline as a fuel extender and an octane enhancing agent, or be used as a neat fuel in internal combustion engines. The utilization of low-cost and renewable plant biomass requires pretreatment of this material to enhance its susceptibility to microbial or enzymic attack, mainly to remove lignin which is a non fermentable component that makes cellulose unavailable for bioconversion [7].

Trichoderma reesi is the most frequently used microorganism as a source of cellulase for saccharification of cellulosic wastes like newsprint. Cellulase is a complex of enzymes containing chiefly endo and exo-glucanases plus cellobiase which rapidly hydrolyze cellulose to glucose. The rate of hydrolysis decreases greatly as the increasingly crystalline portions of the cellulose are attacked [8].

Trichoderma reesi is known to be one of the best producers of cellulase. Other cultures like Trichoderma viridie, Pestalotiposis westeaijiki, and Aspergillus niger are also capable of producing large amounts of cellulase. However, all these cellulases still lack enough of cellobiase or B-glucosidase, an enzyme responsible for hydrolyzing

cellobiose to glucose.

Yeast cultures, particularly Saccharomyces cereviciae, have been the most extensively and widely used microorganisms for efficient conversion of glucose to ethyl alcohol and carbon dioxide. Recently, bacterial cultures of Bacillus and Clostridium species have also been explored for high temperature ethanol fermentation processes [9].

Trichoderma reesi cellulase and cellobiase were used for the saccharification of newsprint to reduced sugar in this study. Yeast culture, Saccharomyces cereviciae, immobilized within calcium-alginate gel matrix was utilized for converting glucose to ethanol and carbon dioxide.

1.4 RENEWABLE FUEL AND ENVIRONMENT:

The state of New Jersey faces a very real problem as to how to dispose of its solid wastes, particularly waste paper. The American Paper Institute recognizes that the disposal of waste paper is becoming increasingly difficult for many local governments in New Jersey. It is more difficult to site landfills, and recent Federal legislations will make land disposal of municipal solid waste [paper] even more difficult and costly [17].

Part of the entire waste paper disposal problem can be solved by recycling. Paper recycling in New Jersey is already a success story. The paper recycling industry is highly efficient, successful industry, made up of 9 paper board mills that use waste paper as their raw material. About 50 % of New Jersey's recoverable paper is collected for recycling. The New Jersey collection rate for recyclable paper is 33 % and is the highest in the U.S.A., which has an average rate of 27 %. New Jersey's rate of collection for recyclable newspaper is 56 % as compared to the national average of 29 % [17].

Paper recycling in New Jersey is a large prosperous and growing business. The major steps involved in the paper recycling process are separation, collection, transportation, manufacturing and sale of recyclable material. The production of good quality paper from waste paper requires deinking or pulp bleaching to obtain the desired brightness. The majority of paper manufacturing or recycling industries use sodium hypochlorite, hydrogen peroxide, and organic detergents in pulp bleaching [18]. In recent years, pulp bleaching processors are turning more and more to hydrogen peroxide for a bright pulp with reduced chemical costs and sizable energy savings [19].

Alternatives to recycling are also being used in an effort to reduce the overall disposal of waste paper. The

various alternatives being implemented are mainly for resource conservation and energy recovery from waste paper.

With the recognition of rapid depletion of petroleum reserves in 1970s, researchers in the U.S. began to update and develop the technology for industrial ethanol production in order to decrease U.S. reliance on imported crude oil for fuels [10].

In 1988, ethanol blended gasoline for automobiles represented 8 % of the total gasoline sales in the United States, accounting for 8.4 billion gallons of ethanol/gasoline blends. Because of its high mixing octane number, ethanol provides motorists with a fuel that produces no discernible difference in a car's performance. Moreover, ethanol blended fuels have proven to decrease carbon monoxide and hydrocarbon tail pipe emissions by 25-30 % [14], thus providing substantial air quality benefits. The most recent studies demonstrate that ethanol blended fuels also reduce urban ozone formation [20].

More than 90 % of the ethanol produced in the United States today is made from corn. This leads toward a potential for rural and agricultural development plus a variety of valuable co-products including high protein animal feed, corn oil, corn meal, and carbon dioxide used to carbonate soft drinks and in making dry ice.

Evidently, a replacement of just 10 % of the nation's

gasoline requirement with an alternate fuel such as ethanol results in a 12.5 % reduction in the nation's crude oil import demands [20].

Therefore, bioconversion of waste paper to sugar and ethanol will help not only in recovering energy and useful byproducts but will also eliminate treatment and disposal of waste water containing organic binders, dyes, and chlorinated solvents produced during the pretreatment of waste paper prior to the recycling process. Bleaching agents like sodium hypochlorite, concentrated hydrogen peroxide, and organic detergents are the best known agents for decolorizing paper pulp in current industrial practices. Also, lignin is shielded with a hemicellulose coating, so an excess of chlorine must be used for bleaching thereby generating a potential hazardous toxic waste which must be properly treated before safe disposal.

The cellulases or hemicellulases can be used to remove hemicellulose and the amount of chlorine needed for bleaching can be significantly reduced, thus lowering its concentration in the effluent. A number of paper mills are now running trials to implement this technique on a large scale to make it cheaper than the bleaching process [16]. However, hydrogen peroxide is the most extensively used bleaching agent in paper making operation and also to improve brightness and reduce reversion during waste paper recycling [19].

1.5 IMMOBILIZATION OF YEAST:

Immobilization refers to confinement of biological agents (enzymes, bacteria, fungi or other microorganisms) into some type of non-toxic water insoluble matirx by means of chemical or physical methods. Each method has its advantages and disadvantages. The physical method includes, adsorption of microbes on a solid phase (like glass beads, polymeric membrane etc.) and physical entrapment in a gel or polymer matrix (like alginate, polyurethane). The chemical method involving chemical bonding has received immense importance in chemical engineering.

By immobilizing the cells into calcium alginate, the cell density of the immobilized system may be much higher than that of traditional suspensions. The immobilization of yeast also offers various advantages during the process as compared to the free system.

The yeast culture, Saccharomyces cereviciae, was entrapped into calcium alginate gel matrix for a variety of reasons such as, easy separation of biomass from the product (ethanol), operational stability, reusability for several times (cost effectiveness), reduced enzyme inhibition, improved resistance to extremes of pH, temperature, concentration of substrate and product formed, and other conditions during the fermentation [13].

CHAPTER II

OBJECTIVES

The primary objective of this research is to evaluate the biocatalytic ability of cellulase and cellobiase in saccharification of newsprint and other waste papers to reduced sugars, and to find the maximum possible conversion of these sugars to ethanol and carbon dioxide using a calcium alginate immobilized yeast fermentation process.

Secondly, an attempt is made here to simultaneously saccharify and ferment the paper slurry to ethyl alcohol in a batch reactor to eliminate the need for separate reactors for hydrolysis and fermentation, and to solve the problem of sugar contamination during the purification.

The objectives of this study were achieved as described briefly in the following steps:

1. Preliminary experiments were conducted to test the biocatalytic ability of cellulase and cellobiase with different kinds of waste feed such as newsprint, computer paper, and copy paper. This work was carried out in small (40 ml) glass beakers, each equipped with a thermometer, magnetic stirrer and heater and stainless steel beads as milling media.

2. The laboratory experiments for the saccharification were carried out by pretreating the newsprint pieces in an attrition bioreactor to obtain fibrous paper slurry. The cellulose slurry was then hydrolyzed to sugar by cellulase and cellobiase for 8 hours of reaction time.
3. Effects of pH, temperature and enzymes concentration on conversion of paper to glucose were studied for a reaction period of 8 hours, a reactor volume of 400 ml, and substrate concentration of 10 g/l in the absence of air-liquid interface.
4. The sugar water, obtained from the enzymic hydrolysis, was purified by centrifugation, filtration and rotary evaporation.
5. Dilute sugar water was fermented to ethanol and carbon dioxide using calcium alginate immobilized yeast at room temperature.
6. An experiment was conducted in a batch reactor for simultaneous saccharification and fermentation of waste paper to ethanol and carbon dioxide using cellulases and immobilized yeast.

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS:

3.1.1 Enzymes:

The selected fungal strain of Trichoderma reesi is known to be the best producer of cellulases and these cellulases are found to be most efficient and extensively used enzymes for catalyzing the break down of cellulose into glucose, cellobiose and higher glucose polymers [11].

The enzymes Celluclast 1.5 L (cellulase) and Novozyme 188 (cellobiase or B-glucanase) were obtained as brown liquid commercial preparations from Novo Nordisk Bioindustrials Inc., Danbury, CT (formerly: Novo Laboratories Inc., Danbury, CT).

A known volume of distilled water was taken in a beaker and its pH was adjusted between 4.4 and 5.3 by adding sodium acetate and acetic acid. Weighed portions of enzymes were then mixed with this liquid to obtain desired enzymic solution. The pH of the enzymic system and the reaction mixture during the experiments was maintained by purging carbon dioxide. Initially, the pH of the enzymatic system was also maintained by sodium acetate and acetic acid during the trial runs, but carbon dioxide for pH control was found more desirable.

3.1.2 Substrate:

Newsprint, like many cellulosic materials, can be considered to consist of cellulose, hemicellulose, and lignin. Cellulose, present in both crystalline and amorphous forms is glucan, while hemicellulose is a mixture of hexans and pentosans. The lignin is shielded with a hemicellulose coating and remains essentially insoluble in the solution; furthermore, it is not biodegradable by cellulase.

An approximate composition (by weight) of newsprint is given in the following table [6],

Table I: Composition of Newsprint:

Dry Analysis	**% Weight**
Cellulose	61.0
Hemicellulose	
xylan	8.0
hexans	8.0
Lignin	21.0
Other	2.0
Total	100.0

The newsprint used in all the experiments was a local newspaper " The Star Ledger " , Newark, NJ. The paper was cut into 1.5 x 1.5 cm pieces for the pretreatment step. As per the discussions with the newspaper manufacturer, the approximate composition of this newsprint was similar to the one given in Table I.

3.1.3 Yeast:

Yeast culture, Saccharomyces cereviciae, is the most common type of microorganism utilized for the production of ethanol from glucose both for research purposes, and commercially and hence was chosen for this study [9].

The active yeast (Saccharomyces cererviciae) used was obtained from Universal Foods Corporation, Clifton, NJ (C/o. Red Star Specialty Products, Milwaukee, WI), and was immobilized into calcium alginate gel matrix.

3.2 PREPARATION OF BIOCATALYST:

3.2.1 Cellulase Preparation:

The active enzyme components of Celluclast 1.5 L are readily soluble in water at all concentrations which occur in normal usage. Their activity, as reported by the manufacturer (Novo Nordisk Bioinustrials), is reported to be optimum in

the range of pH between 4.5 and 5.5, temperature between 25 and 50^{o} C. At lower temperatures (5-10^{o} C), the shelf life is considerably increased.

For experimental purposes, a known portion of cellulase was dissolved in a known volume of distilled water at specified pH, by purging CO_2 gas. This was followed by dissolving a known amount of cellobiase in the same solution. Cellulase and cellobiase in weight ratio of 5:1 were used in all the experiments.

3.2.2 Preparation of Immobilized Yeast:

In this study, 50 g of dry yeast and 2.5 g of NaCl were added to a blender containing 250 ml of water, and thoroughly mixed for 2-3 minutes to create a uniform suspension. Another 220 ml of water was added to this mixture followed by addition of 3.75 g of alginic acid, and the paste was allowed to mix for 2-3 minutes. The paste was then extruded dropwise in a 0.1 molar calcium chloride solution to obtain uniform immobilized yeast beads. These beads were suspended in tap water and then stored between 0-5^{o} C and were used for fermentation from time to time. A schematic of the installation is as shown in Appendix A.

3.3 ANALYSIS:

3.3.1 Reducing Sugars:

The reducing sugars were analyzed by the dinitrosalicylic acid (DNS) assay as described by Miller [1]. The DNS reagent was prepared by dissolving 1 g DNS acid, 0.2 g of phenol, 0.05 g of Na-sulfite, and 1 g of NaOH in 100 ml distilled water to obtain a 1 % DNS reagent. Twenty grams of rochelle salt (Na-pottasium tartarate) was dissolved in 50 ml distilled water to get 40 % rochelle salt solution which was used with DNS reagent to impart color stability to the sugar solution after reaction with DNS reagent.

A 3 ml sample of sugar solution was taken in a 14 mm test tube and 3 ml of DNS reagent was added to it. The mixture was then heated for 15 minutes in a boiling water bath in order to develop the color (brown). To this 1 ml of 40 % rochelle salt was added immediately and the solution was allowed to cool to ambient temperature. The color intensity was measured using UV-Visible Spectrophotometer (Spectronic 20, Bausch and Lomb).

For this study, a calibration plot of absorbance (ordinate) versus glucose concentration in mg/100 ml (abscissa) was drawn at a wavelength of 575 nm. The concentrations of unknown sugar solutions were then determined using the linear [regression] calibration curve.

3.3.2 Ethanol:

The bio-ethanol was detected by gas chromatography (Shimadzu GC-8A) using a thermal conductivity detector (TCD) and helium as the carrier gas. A stainless steel column (1 m long, 2.3 mm i.d.) packed with Porapak N (80-100 mesh), was used to elute ethanol at an oven temperature of 150^{o} C. The Chromatopac, Shimadzu C-R3A integrator was used to calculate the peak area [12].

For calibration purposes, a plot of peak area (ordinate) versus concentrations of water, ethanol and acetic acid by volume (abscissa) was drawn. The concentrations of unknown samples were determined using the linear [regression] calibration curve.

3.3.3 Measurement of Saccharifying Cellulase Activity:

The activity of Celluclast 1.5 L (cellulase) was determined using the Filter Paper Activity test (FPA) of Mandel et al. [8]. The activity was expressed as IU/g of enzyme. One International Unit (IU) is the amount of enzyme, which under standard conditions, degrades cellulose to reducing sugars with a reduction power corresponding to 1 u-mole glucose/minute.

The filter paper activity of cellulase was measured by, reacting 5 mg/ml of cellulase at pH 4.8 in 0.05 M Na-acetate solution with 1 x 6 cm Whatman # 1 filter paper (50

mg) in 18 mm test tube at 50° C for 1 hour; subsequently, a 3 ml of DNS reagent was added to stop the reaction and the test tube was placed in boiling water bath for 15 minutes for color development. One ml of rochelle salt solution was also added to this mixture for color stability. The mixture was then cooled to room temperature and the color intensity was measured using UV-Visible Spectrophotometer at 575 nm to determine the amount of sugar produced.

As per the Mandel et al. [8] procedure, the initial cellulase concentrate had an average FPA activity of 262.93 IU/g of cellulase or 315.51 IU/ml of cellulase.

3.3.4 Activity of Immobilized Yeast:

The activity of immobilized yeast, entrapped in calcium alginate gel matrix, was tested in order to obtain maximum possible conversion of glucose to ethanol during the fermentation.

The activity of immobilized yeast was measured using a microassay reactor and a strip chart recorder. The microassay reactor consisted of 1.8 ml glass jacketed vessel, and was equipped with water circulation system, a temperature controller and a Clarke type dissolved oxygen probe for measuring the dissolved oxygen consumption.

The refrigerated immobilized yeast beads were shocked in water at 40-45° C for 10 to 15 minutes before performing

the test. About 0.14 g beads were then dropped into the microassay reactor containing 1.6 ml of 1 % glucose solution saturated with oxygen. The oxygen consumption value was recorded on a strip chart recorder.

The immobilized yeast had an average activity of 0.102 n-moles oxygen/min-mg of yeast.

3.4 EXPERIMENTAL PROCEDURE:

The process consisted of three steps: pretreatment of the newsprint to cellulose slurry in an attrition bioreactor, enzymic hydrolysis of cellulose to glucose by combined cellulase and cellobiase system, and fermentation of glucose to bioethanol using immobilized yeast.

The biocatalytic ability of commercial preparations of cellulase (Celluclast 1.5 L) and cellobiase (Novozyme) to hydrolyze cellulose to glucose was tested by conducting preliminary trial runs in a small reactor (40 ml) with different types of waste paper. The cellulose content of newsprint was reported to be 60 % by weight of newsprint [6].

3.4.1 Testing the Biocatalytic ability of Enzymes:

Preliminary experiments were conducted on a trial basis to study the behavior of enzyme(s) during the saccharification process. The waste paper, from computer printouts and copying machines was selected for these trials. The waste paper was cut into 1 x 1 cm pieces and manually crushed in a mortar with water to make cellular slurry.

Known amounts of enzymes, cellulase and cellobiase in a weight ratio of 5 : 1, were dissolved in known volumes of water at pH between 4.5-5.0. Initially, the pH of the solution and the reaction mixture was maintained using sodium acetate and acetic acid. Later, carbon dioxide bubbling was found to control pH adequately. Next, 10 ml of 10 mg/ml paper slurry was heated to a desired reaction temperature by a heater with magnetic stirrer, and small amount of 3/16 in. stainless steel balls as milling media. Once the desired temperature was reached, 10 ml of 4 mg/ml mixed enzyme solution was added to this and the full reactor was completely sealed from the top by parafilm in order to eliminate the air-liquid interface, which deactivates the enzymes. The reaction was carried out in a time range of 0 to 8 hours, pH between 4.5 and 5.0 and temperature between 45.0 to 52.0^{o} C with moderate stirring. Samples were withdrawn from the reactor every hour and were tested for sugar by a colorimetric method as described by Sigma [2].

After running a series of trial runs under different operating conditions and making careful observations, as discussed in Chapter 4, the waste newsprint was converted to glucose. These trial runs were conducted to check the feasibility of the enzymic process.

3.4.2 Pretreatment of Newsprint:

Recent studies have revealed that the pretreatment of waste newspaper into very small fiber particles of 1 mm or less by attrition using stainless steel beads followed by enzymic hydrolysis significantly increases the rate and extent of saccharification [3]. The milling action generates new surfaces and creates an easy ground for the enzymes to cleave glucosidic bonds, consuming available reaction sites and further weakening the cellulose structure.

The attrition bioreactor was used in a pretreatment step for preparing fine cellulose slurry from waste paper, as shown in Figure 1. It consists of a stainless steel vessel, milling media, and a marine type impeller. Mixing is provided by a variable speed agitator driven by a 1/3 hp motor (Lightnin, Ohio). The impeller is located 0.5 inch above the bottom of the vessel. This allows free movement of the balls under the impeller. The milling media is 3/16 in. SS-302 stainless steel burnishing balls.

The waste newsprint was cut into 1.5 x 1.5 cm pieces

for the pretreatment step. The weighed portion of paper pieces were crushed to a very fine cellulose slurry in the ABR using a known amount of water. For a given batch the grinding time was one hour. The fibrous paper slurry was stored in the refrigerator between 0-5^{o} C and was used in all the experiments.

3.4.3 Deinking of Newsprint:

The waste (printed) newsprint containing dyes, organic binders, ash, lignin, and other unknown substances was washed with a mixture of Phloroglucinol (1,3,5-Trihydroxybenzene), 30 % Hydrogen peroxide or Na-hypochlorite, Tamol, and 1 N HCl in equal proportion by weight for 10-15 minutes for decolorizing the newsprint and to remove the lignin. Hydrogen peroxide was used for bleaching purposes, Tamol SN 6-0224 (Rohm and Haas, Philadelphia, PA) as an dispersing agent, and Phloroglucinol was added to remove the lignin.

Several attempts were made with proper mixing to decolorize the newsprint, and although lignin was liquefiable, no appreciable amount of ink could be removed. Instead, the newsprint was found to disintegrate into small fibers. The removal of lignin, dyes, binders, and pigments from newsprint by decolorization may have increased the percent conversion of paper to sugar.

3.4.4 Saccharification of Cellulose:

The bioreactor consisted of a 500 ml jacketed glass vessel equipped with a magnetic stirrer, a heating coil with a temperature controller and a pH probe. The reactor was completely sealed from the top with parafilm in order to eliminate air-liquid interface which inhibits the enzyme activity. Samples from the reactor were withdrawn every hour, spun in a centrifuge and the clear sugar water was tested using the DNS reagent. The pH of the reaction medium was maintained by periodically bubbling carbon dioxide gas through the reactor. Carbon dioxide was generated in a separate reaction between sodium bicarbonate (NaHCO3) and dilute sulfuric acid, as shown in Figure 2.

At the end of 8 hours, the mixture was cooled to 0^{o} C using ice to stop the reaction. The chilled mixture was then centrifuged at 0^{o} C and a clear sugar water was obtained after filtration. The dilute sugar solution (gray in color) obtained after filtration was concentrated with a rotary evaporator between 60-70^{o} C. The dilute sugar solutions, obtained after centrifugation and filtration, in the range of 280-300 ml were concentrated to about 30-50 ml volumes, during which more impurities were concentrated. The pH of concentrated sugar solution was found to decrease from 5.0 to 3.0.

The unconverted paper after 8 hours of enzymic

hydrolysis was obtained as a pellet from centrifugation, during the separation of sugar water from the reaction mixture, and was oven dried (after filtration) at about 70° C for 4 to 5 hours. The dry-hard paper was weighed and the amount of paper consumption calculated.

3.4.5 Fermentation of Glucose to Ethanol and Carbon dioxide:

In the final step, the concentrated sugar water was fermented in a 250 ml sealed conical flask at room temperature using immobilized yeast beads.

In one experiment, 50 grams of immobilized yeast beads were shocked between 40-45° C in a beaker containing tap water for 10 to 15 minutes with stirring. About 0.5 g of d-glucose was fed to these beads to activate them quickly. The activated yeast beads were then washed several times with tap water, and the activity was measured.

Next, 50 g of immobilized yeast were put into an Erlenmeyer flask equipped with a magnetic stirrer. To this, 35 ml of sugar water was added for fermentation of glucose to ethyl alcohol and carbon dioxide was liberated as a byproduct. Fermentation was carried out until the completion of reaction where CO_2 gas stopped emerging. The flow rate of carbon dioxide formed during the fermentation was measured by a soap-bubble meter. Bio-ethanol formed by fermentation was separated and purified from the ethanol-water mixture by

batch distillation, and was analyzed by gas chromatograph.

3.4.6 Simultaneous Saccharification and Fermentation (SSF):

The two separate processes, enzymic hydrolysis and fermentation can be combined by using a mixed batch reactor. Here, simultaneous saccharification and fermentation provide a synergistic effect, with enzymes hydrolyzing cellulose to glucose, and at the same time, yeast breaking down glucose to ethanol and carbon dioxide.

The direct production of alcohol from cellulose in an SSF process alleviates the problem of sugar contamination during the purification step as well as eliminates the need for separate reactors for each process individually.

In this study, a thick concentrated paper slurry (20.1 g/l) of 400 ml was charged in a 750 ml Erlenmeyer flask equipped with a magnetic stirrer, heating coil and temperature controller, and a pH probe. A reflux condenser with a soap bubble meter was also attached to the reactor to prevent the loss of ethanol vapors, arising from SSF at higher temperature. Carbon dioxide gas was purged through the slurry to maintain a pH of 4.6 and the reactor was then heated to a reaction temperature of 40^{o} C. The reactor assembly of the SSF process is schematically represented in Figure 3.

A combined enzymic system, consisting of cellobiase

and cellulase in the ratio 1:5 by weight, at 6 g/l was mixed with the paper slurry followed by addition of 97 grams of immobilized yeast beads in a 750 ml bioreactor. The reactor was closed from the top and was completely sealed using parafilm. The bioreaction was continued for seven [7] hours during which the formation of CO_2 gas was observed through the soap bubble meter.

At the end of 7 hours of reaction period, the mixture was chilled to $0^{o}C$ using ice to stop the reaction. The chilled mixture was separated from the obtained products by filtration and spinning the product mixture of ethanol-sugar water to remove any suspended particles. The product mixture was then analyzed separately for glucose and bioethanol by the DNS reagent method [1] and gas chromatography [12] respectively. The results obtained from the SSF process are discussed in detail in Chapter 4.

CHAPTER IV

RESULTS AND DISCUSSION

In this study we investigated the effects of pH, temperature and enzymes concentration on conversion of waste paper to glucose by combined enzymic system of cellulase and cellobiase in the absence of an air-liquid interface without using stainless steel beads.

4.1 BIOCATALYTIC ABILITY OF ENZYMES ON WASTE PAPER:

These experiments show that about 83 % conversion of waste paper (from computer printouts) to sugar is achieved in a reactor volume of 20 ml, enzymes concentration of 4 mg/ml, a substrate concentration of 10 mg/ml, and pH of 4.5 at 47.0^{o} C for 8 hours of reaction period [Figure 8].

It was found from the paper manufacturer that the cellulose content of "Xerox Computer Paper" was about 85-90 % by weight. Thus, the higher the amount of cellulose present in waste paper, the higher will be the percent conversion of paper to glucose.

4.2 EFFECTS OF pH, TEMPERATURE AND CONCENTRATION OF ENZYMES ON CONVERSION OF NEWSPRINT TO REDUCED SUGAR:

Initially a pH of 4.6 was selected on the basis of discussions with the enzyme manufacturer [Novo Nordisk Bioindustrials]. Next, the temperature was varied from 40.0°C to 50.0°C. This indicates a local optimum at 45.0°C, as shown in Table II.

Subsequently, the temperature was kept constant at 45.0°C, and the pH was varied from 4.4 to 5.3. Again, an optimum was found at pH 4.6, as shown in Table III.

The temperature of 45.0°C and pH of 4.6 are only local optima, and not necessarily the best conditions. Further experiments would be required if global optima were desired.

Nonetheless, enzymes concentration was varied keeping temperature at 45.0°C and pH at 4.6. The results are shown in Table IV, indicating a maximum conversion at from 3 to 4 g/l.

Table II: Effect of pH on Conversion at 45.0°C*

pH	% Conversion g sugar/g paper
4.40	50.01
4.60	51.95
4.85	50.57
5.15	49.73
5.30	47.18

Table III: Effect of Temperature on Conversion at pH of 4.6*

Temperature	% Conversion g sugar/g paper
40.0	49.45
43.0	53.69
45.0	55.09
47.0	45.77
50.0	48.88

* Reaction conditions: 400 ml volume, 8 hours reaction time, 4 g/l enzymes concentration, and 10 g/l starting paper concentration.

Table IV: Effect of Concentration of Enzymes on Conversion at 45.0°C, pH of 4.6, 400 ml reactor volume, 8 hours of reaction time, and 10 g/l of starting paper concentration.

Enzymes g/l	% Conversion g sugar/g paper
1.0	41.87
2.0	44.36
3.0	53.52
4.0	53.69
5.0	50.01

At an enzymes concentration of 3 g/l, the amount of sugar produced was 1.520 g, while at a concentration of 4 g/l, the amount of sugar produced was 1.584 g. A 4.0 % increase in glucose production may not be significant when compared to the increase in enzymes concentration by 1 g/l. The results for the effects of pH and temperature agree well with previous studies conducted by Jones and Lee [3].

From Figures 4, 5, & 6, on an average 38.5 % conversion of paper to sugar is calculated for the first hour of reaction. As can be seen from Table V, as well as Figures 4-6, the reaction proceeds slowly during the remaining period of seven hours.

Table V: Enzymatic Hydrolysis of Newsprint

Time hr	pH	Temperature ^{o}C	Sugar g/l	% Conversion g sugar/g paper
1	4.40	45.0	3.763	37.63
2	4.35	45.0	3.837	38.37
3	4.45	45.5	3.995	39.95
4	4.40	45.0	4.142	41.42
5	4.35	45.5	4.176	41.76
6	4.40	46.0	4.266	42.66
7	4.40	45.0	4.436	44.36
8	4.40	45.0	5.001	50.01

The work carried out by Jones and Lee [3] indicates that on an average, 17.0 % conversion of paper to sugar was achieved during the first hour of combined milling and enzymic hydrolysis of newsprint using an ABR under standard operating conditions. This clearly shows that higher reaction rates are achievable using a one hour pretreatment step followed by one hour of saccharification in a simple stirred batch reactor with appreciably low power consumption.

In our study, on an average, 50.0 % conversion was achieved in 8 hours of reaction period with most of the conversion (38.5 %) taking place in the first hour. The reason behind achieving only 50.0 % conversion of paper to glucose is unknown. The maximum conversion achievable for a typical paper containing 60 % cellulose is about 67 % (on paper basis) because of the water of hydrolysis. This conversion is not attained, possibly due to enzyme(s) deactivation, inhibition or low cellulose concentration as the reaction proceeds. These speculations need to be investigated. In order to obtain higher conversion, it might be worth injecting another shot of substrate after one hour of reaction rather than continuing the batch for 8 hours, provided that the enzymes are active.

4.3 STIRRED BATCH REACTOR:

The pretreatment of newsprint using attrition followed by enzymic hydrolysis in a stirred batch reactor under specified conditions produced a maximum conversion of 55 % within 8 hours of reaction period. Jones and Lee [3] also reported that about 55.0 % conversion was achieved in 6 hours of simultaneous attrition and reaction in the ABR. The present study shows that one hour of pretreatment step followed by enzymic hydrolysis of 8 hours in a stirred batch reactor produces effective results. Deeble and Lee [4] have reported that enzyme deactivation increases with increasing power input. Considering the elimination of milling media, the stirred batch reactor studied here produced effective results with very low power consumption, and hence less enzyme deactivation.

4.4 USE OF CELLOBIASE WITH CELLULASE:

Cellobiose is usually formed during the saccharification of cellulose by cellulase. Since, cellobiose is not a fermentable sugar, cellobiase was added to cellulase to obtain complete conversion of cellulose to fermentable sugar. The use of cellobiase with cellulase in a ratio of 1:5

by weight, as recommended by Novo Nordisk Bioindustrials Inc., resulted in maximum achievable conversion for enzymes concentration of 4 g/l [21].

Jones and Lee [3] studied the enzymic hydrolysis using one enzyme (only cellulase) and obtained about 32.0 % conversion in 6 hours of reaction period. In another experiment they used mixed enzyme system containing equal amounts of cellulase and cellobiase and achieved 45.0 % conversion of paper to sugar with similar operating conditions.

4.5 FERMENTATION OF GLUCOSE:

The yeast ethanol fermentation is known to be the most efficient pathway for ethanol production from glucose [9]. The sugar solution obtained from the hydrolysis of newsprint, was degraded to ethyl alcohol and carbon dioxide by immobilized yeast in a volumetric flask at 25^{o} C both in dilute and concentrated forms.

A dilute sugar solution of 35 ml (4.35 mg/ml) at pH of 5.0 was fermented by 50 g of immobilized yeast to ethanol and carbon dioxide, and about 89 % conversion of sugar to ethanol and carbon dioxide was obtained. Figure 7 shows a typical fermentation curve for the production of carbon dioxide.

For the case of sugar solution with pH 3.0, about 16-23 % of sugar was converted to ethanol and carbon dioxide. This shows that the amount of sugar consumed during the fermentation at pH of 3.0 was low as compared to that at pH of 5.0.

4.6 CONTAMINATION OF REDUCED SUGAR:

The sugar water resulting from enzymic hydrolysis of newsprint was gray in color with small amount of dissolved impurities even after centrifugation followed by filtration. These impurities are contributed from the dye, organic binders and bleaching agents, lignin, hemicelluloses and other materials used in newsprint manufacturing and printing operations.

The diluted sugar water obtained after filtration was concentrated with a rotary evaporator between 60-70^{o} C, during which more impurities were concentrated, and appeared as black suspended particles. The separation of these particles by filtration did not change the color of sugar solution. A dilute sugar solution of 323 ml (4.14 mg/ml), having 1337 mg of sugar, was concentrated to 40 ml by rotary evaporation. The concentration of 40 ml of concentrated sugar water was found to be 4.38 mg/ml, having 175 mg of sugar.

Thus, about 87 % loss of sugar, from 1337 mg to 175 mg, was observed during the entire purification step. The pH of concentrated sugar solution was found to decrease from 5.0 to 3.0.

The sugar contamination may be due to some side reaction(s) taking place between sugar and unknown dissolved impurities during the removal of excess water from dilute sugar solution by rotary evaporation. Therefore, use of any performance based sugar purification method (i.e. activated carbon adsorption, recrystallization, ultrafiltration) is recommended to prevent the contamination and loss of sugar. We also recommend to use concentrated paper slurry in saccharification process to get concentrated sugar solution which can be easily fermented to ethanol and carbon dioxide, thus helping in eliminating the evaporation step used to concentrate the dilute sugar solution.

4.7 CONVERSION OF PAPER TO ETHYL ALCOHOL:

Based on the experiments, we found that 1.721 g of sugar was obtained from 4.0 g of newsprint hydrolyzed by enzymes under the specified operating conditions, giving a yield of 43.02 %. Considering 89 % conversion of sugar to ethanol and carbon dioxide by fermentation, 1.721 g of the

reducing sugar gives 0.6 g alcohol, with 80 % fermentation efficiency. Thus a maximum of 0.6 g of ethanol is obtained from 4.0 g of newsprint used, under specified reaction conditions.

4.8 SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF):

An experiment for simultaneous saccharification and fermentation of newsprint to ethanol and carbon dioxide was also conducted to save time, energy, and to improve the process. In the course of 7 hours of reaction period at 40.0^{o} C, and pH of 4.6, we found that only 6.032 % of the paper was converted to sugar and only 10.34 % of this sugar was converted to ethanol and carbon dioxide. Evidently, about 9 fold decrease in conversion of paper to glucose and about 8 fold decrease in fermentation of sugar to ethanol and CO_2 occured during the SSF, as compared to a separate hydrolysis and fermentation processes.

This drastically smaller conversion of both paper to sugar, and sugar to ethanol can be due to several reasons, which need to be investigated. The possible reasons for low conversion(s) can be due to the presence of ethanol, formed by fermentation of sugar, inhibiting the enzymes cellulase and cellobiase responsible for the hydrolysis of paper to

sugar, and yeast inhibition due to ethanol production. It might be possible that one of the systems, either yeast or cellulases, inhibited the other one by a definite action, causing irreversible damage. Another reason for such a low conversion may be due to the temperature effects on both cellulases and yeast, since each one of them have their highest activity at different temperatures.

4.9 PROCESS EVALUATION:

The study conducted is in support of a technique of biomass conversion of solid waste (newsprint) into a clean fuel (ethanol) on the laboratory scale. The next step would be to scale it up to a pilot level. Factors such as enzyme availability and its recycling, prevention of sugar contamination during purification, energy requirements for the purification of bioethanol by distillation and formation of carbon dioxide for maintaining pH during hydrolysis, and the cost of carbon dioxide collection during fermentation are to be considered. A detailed economic analysis of these factors needs to be evaluated for any practical importance. A proposed process flowsheet is shown in Figure 9.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS:

5.1 CONCLUSIONS:

Bioconversion of newsprint and waste paper to glucose using enzymatic hydrolysis by cellulase and cellobiase was found to be a successful process. The enzymatic hydrolysis of waste paper by a mixed function preparation of cellobiase and cellulase in the absence of air-liquid interface improved the catalytic ability of the enzymes and coordinated towards higher percent conversion of cellulose to reduced sugar. Ethanol fermentation of glucose by immobilized yeast facilitated the production of ethyl alcohol from sugar and simplified the problem of product separation, enzyme inhibition, and initiated the reuse of yeast for several times (cost effectiveness).

Considering the overall performance of cellulase, cellobiase, and immobilized yeast for bio-transformation of waste paper to fuel (energy), the following important conclusions were drawn:

1) The effects of pH, temperature, and enzymes concentration under specified process conditions showed that the mixed enzymic system influenced the overall conversion of paper

to sugar and also increased the rate.

2) Experiments on enzymic hydrolysis of newsprint by cellulase and cellobiase, after pretreatment in an ABR, have shown that about 38.5 % conversion was obtained in one hour reaction time and the reaction then proceeded slowly for the remaining seven hours.
3) Bioconversion of paper slurry to sugar was highly efficient without using stainless steel beads, and this helps in power saving.
4) The increase in enzymes concentration beyond 3 g/l did not increase the conversion of paper to sugar significantly. Hence, the enzymes concentration of 3 g/l can be considered as optimum for 10 g/l of paper concentration.
5) The fermentation of reducing sugar by immobilized yeast indicates that higher conversion of sugar to ethanol is achievable at pH of about 5.0 than that at pH of 3.0.
6) The contamination of sugar, during the entire purification step, resulting in low pH (3.0) sugar solution, could be the primary reason for the lower conversion of sugar to ethanol by fermentation.

5.2 RECOMMENDATIONS:

5.2.1 Saccharification:

The commercial preparation of cellulase (Trichoderma reesi) costing $ 60/kg, is substantially high and therefore, we recommend the use of a potential hybrid bioreactor for an in-house production facility of extracellular cellulase from selected mutants of Trichoderma reesi. Also enzyme recycling is suggested for active enzymes.

The sugar losses due to the contamination occurring from the purification step may be prevented by using a better performance based sugar concentration and purification method.

Use of any suitable deinking method for newsprint prior to the pretreatment step followed by saccharification is strongly recommended.

5.2.2 Fermentation:

For ethanol fermentation, the main obstacle is non-availability of glucose. While we observed that the obtained sugar concentration was partially effected by factors such as pH, temperature, and concentration of enzymes, we recommend a bioreactor design of a membrane configuration as discussed by Lakhwala of the NJIT Biotechnology Laboratory [15]. The membrane bioreactor can also be utilized for quick fermentation of sugar to ethanol.

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Figure 1. Attrition Bioreactor (ABR)

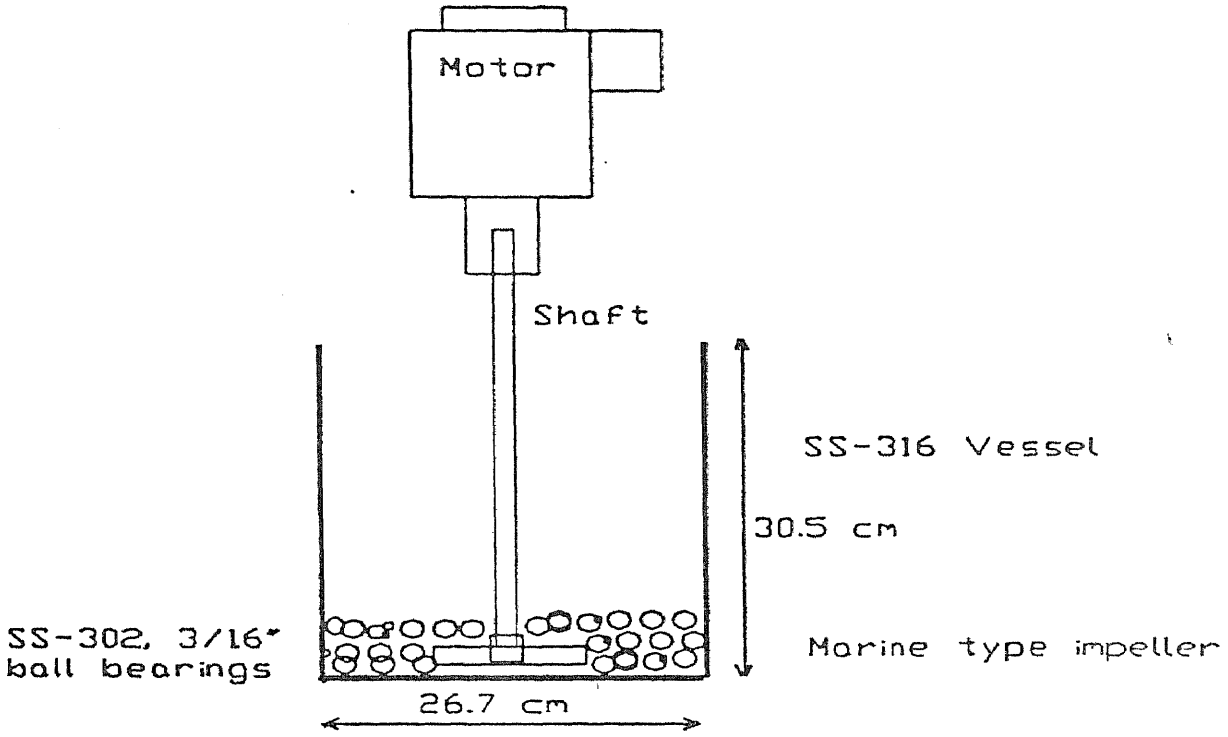


Figure 2. Stirred Batch Reactor

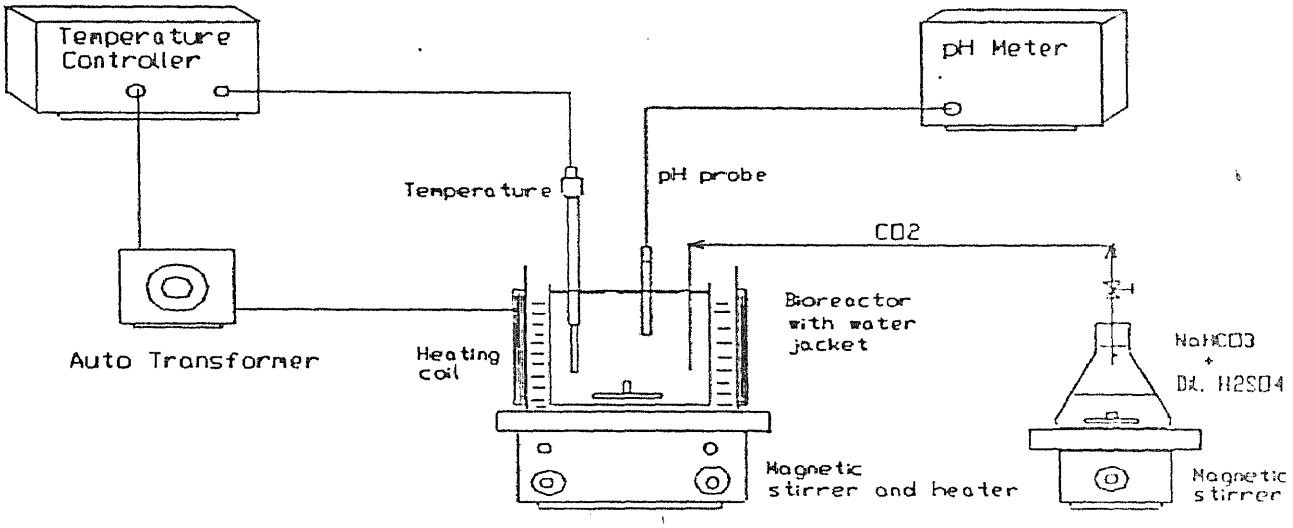
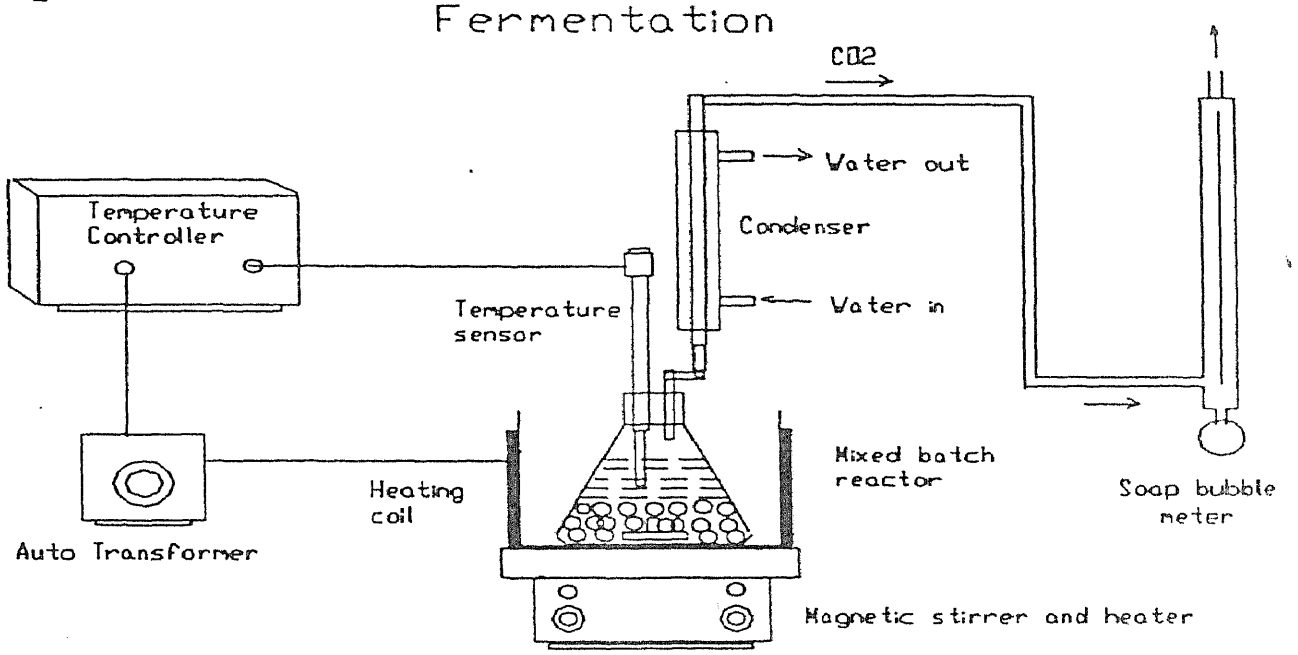


Figure3. Simultaneous Saccharification and Fermentation



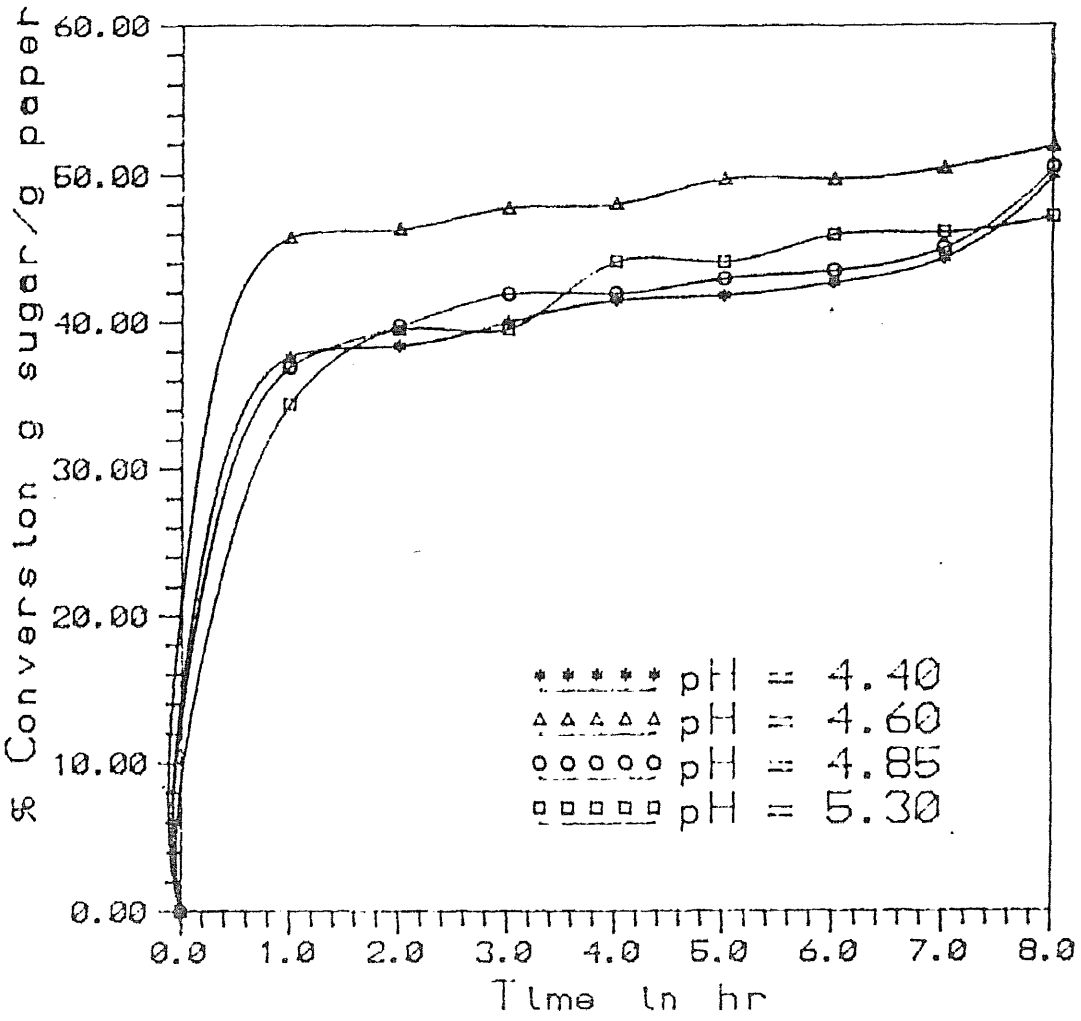


Figure 4: Effect of pH on conversion
Reaction conditions: T = 45.0 C, Cs = 10 g/l, Ce = 4 g/l
reactor volume = 400 ml, reaction period = 8 hrs

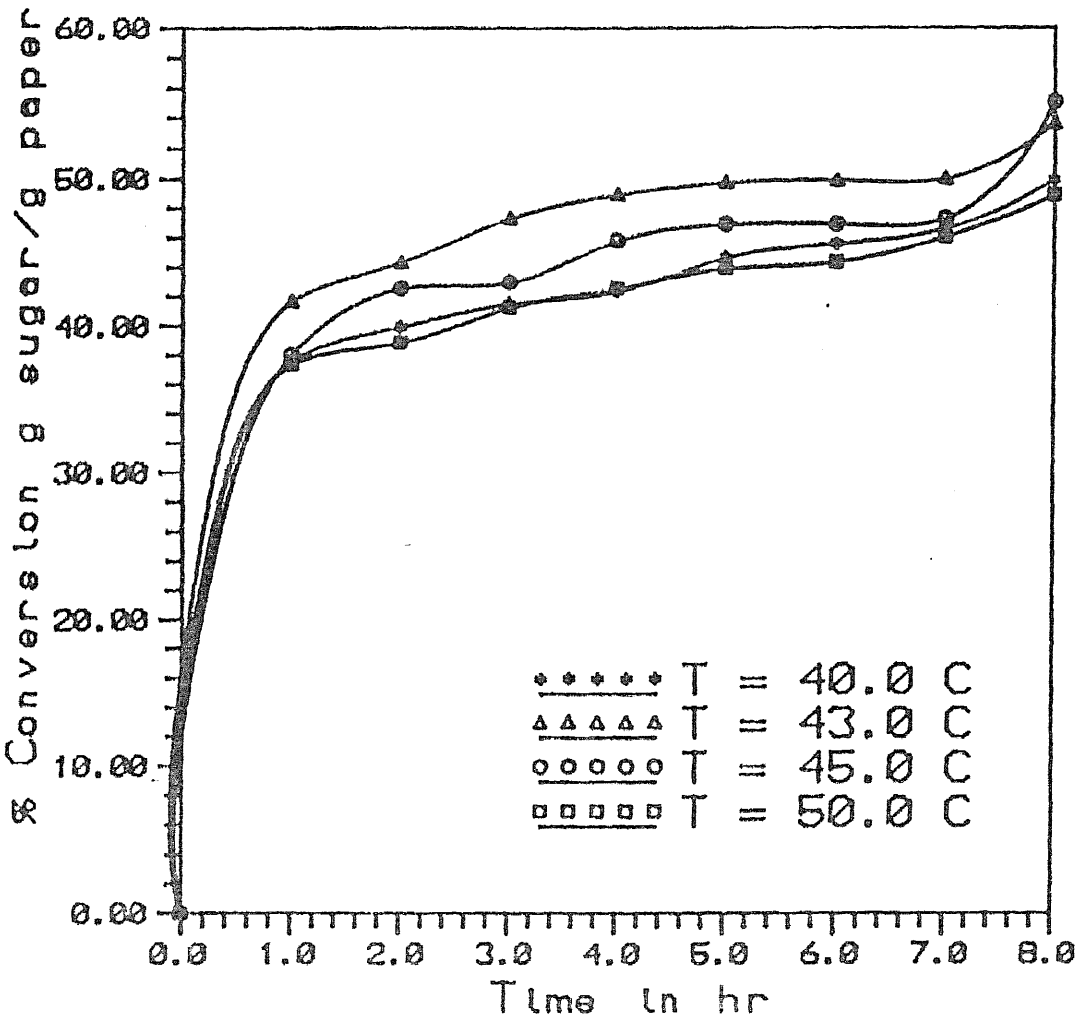


Figure 5: Effect of temperature on conversion
Reaction conditions: pH = 4.6, Cs = 10 g/l; Co = 4 g/l
reactor volume = 400 ml, reaction period = 8 hrs

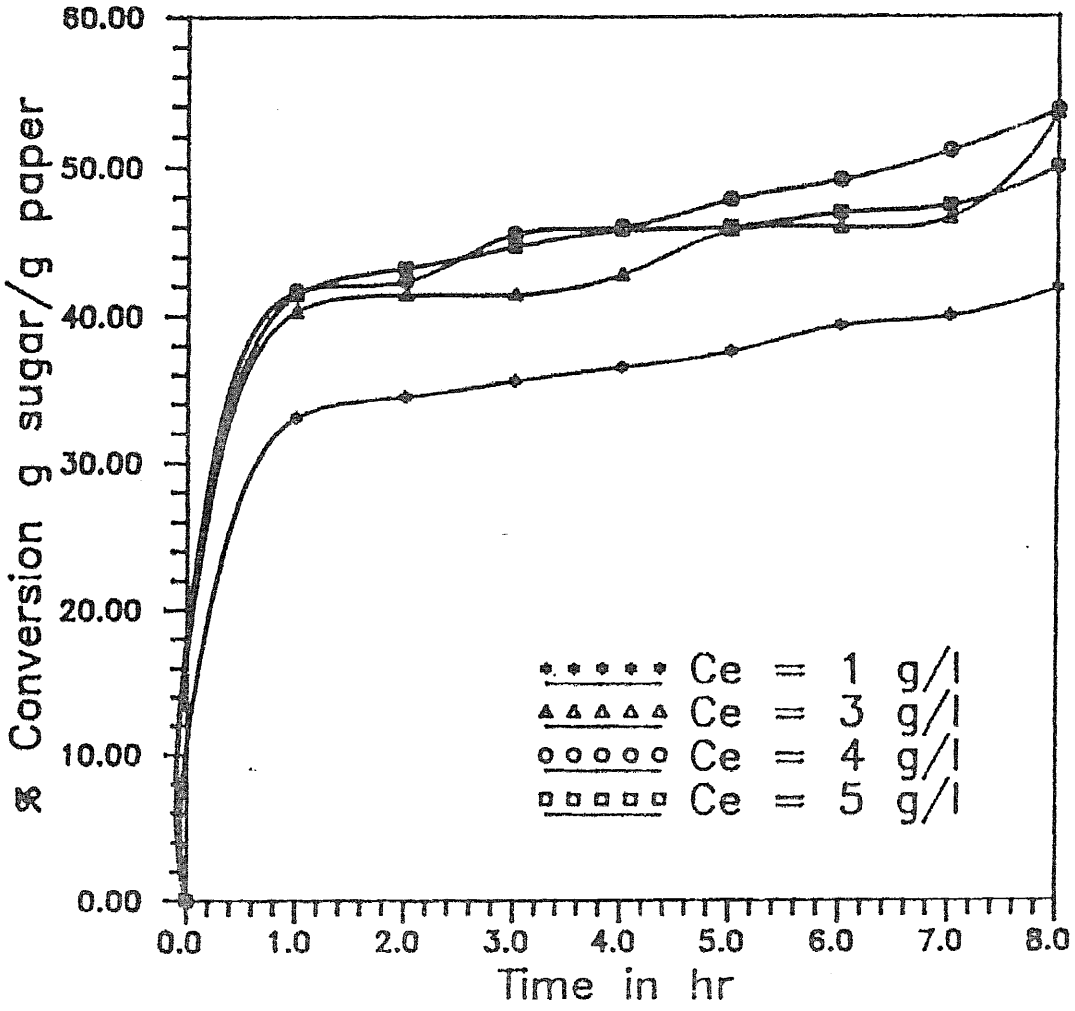


Figure 6: Effect of enzymes concentration on conversion
Reaction conditions: T = 45.0 C, pH = 4.60, Cs = 10 g/l
reactor volume = 400 ml, reaction period = 8 hrs

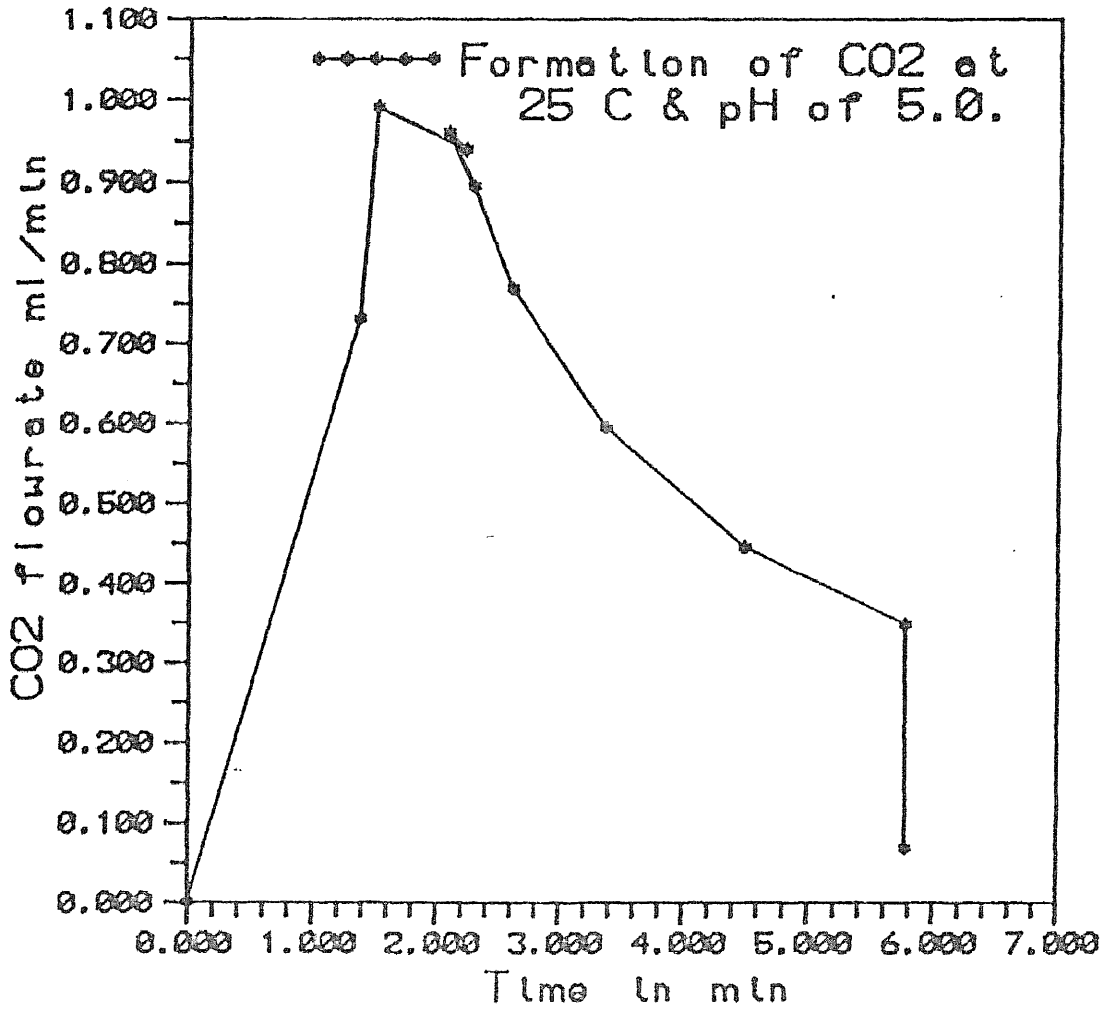


Figure 7. Fermentation of glucose to ethanol and CO_2 using immobilized yeast.

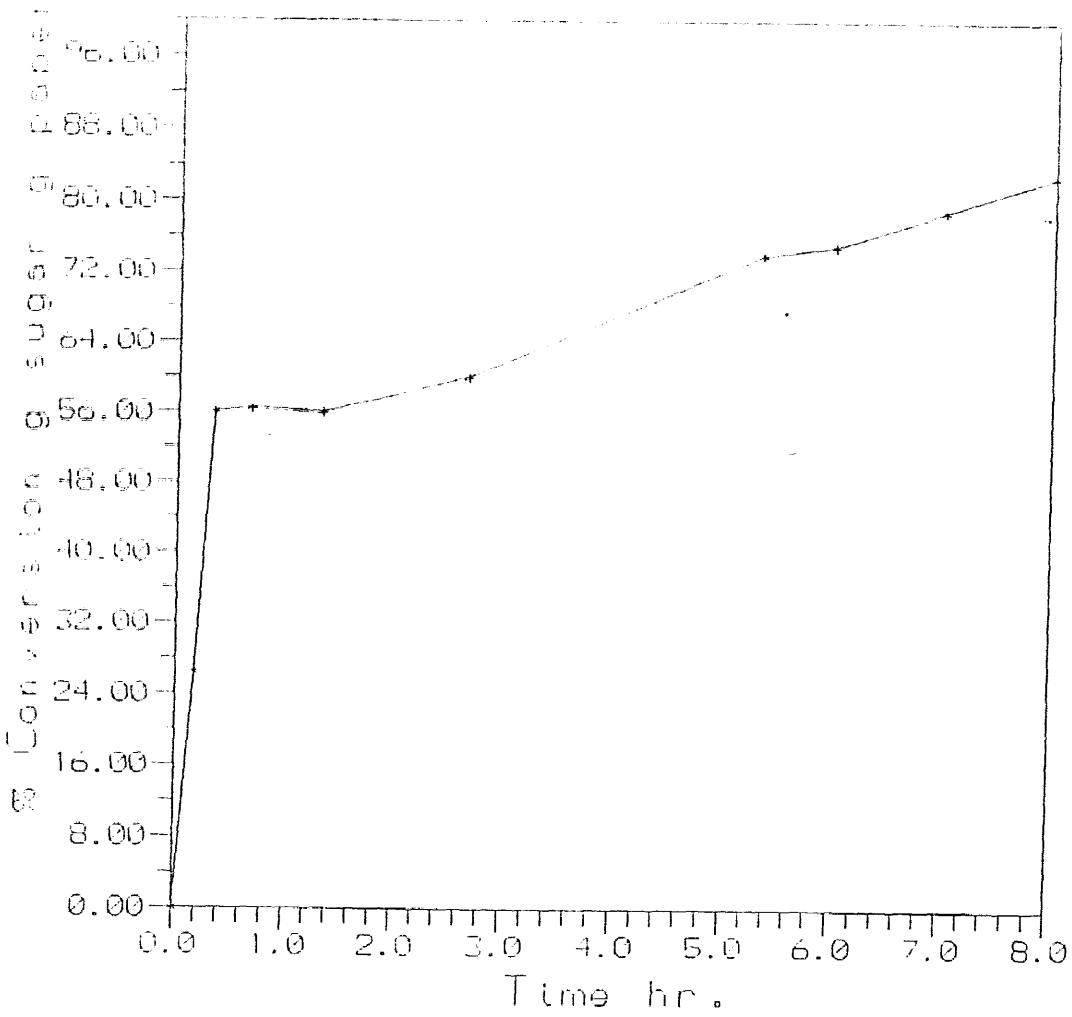
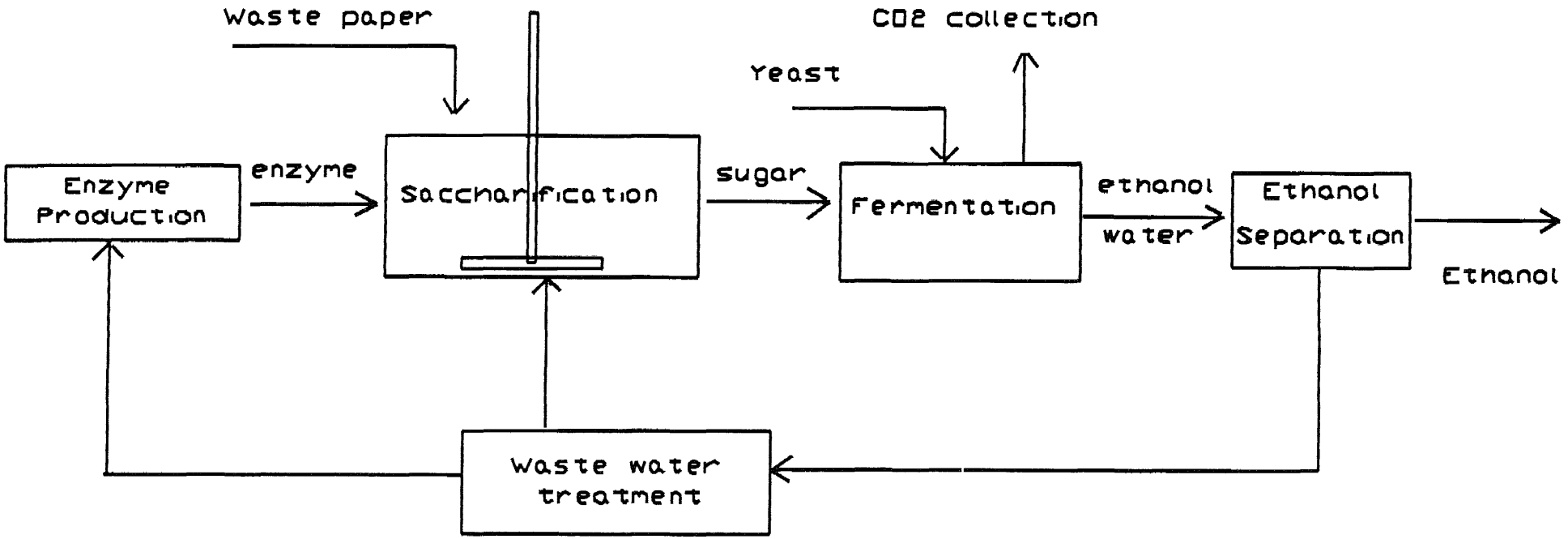
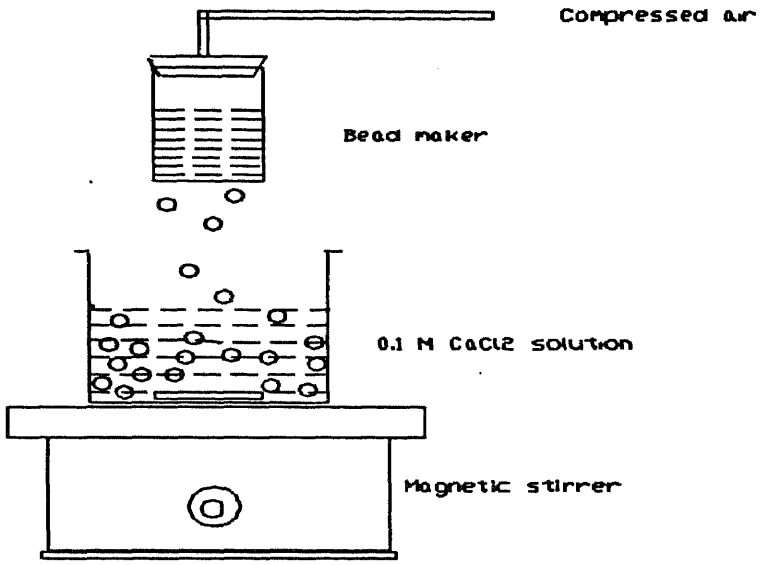


Fig.8 Enzymatic hydrolysis of waste (computer) paper
pH = 4.5, T = 47.0 C, reactor volume = 20 ml
C paper = 10 g/l, C enzymes = 4g/l

Figure 9. Proposed Process Flowsheet





Bead making process. An alginate-yeast mixture dropped through bead maker in an 0.1 M CaCl2