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Development of a biofilm bioreactor for enhanced ethanol production

Mahipal Reddy Kunduru
Iowa State University

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**Development of a biofilm bioreactor for enhanced ethanol
production**

Kunduru, Mahipal Reddy, Ph.D.

Iowa State University, 1994

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Development of a biofilm bioreactor for enhanced ethanol production

by

Mahipal Reddy Kunduru

**A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY**

**Department: Food Science and Human Nutrition
Major: Food Science and Technology**

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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For the Major Department

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For the Graduate College

**Iowa State University
Ames, Iowa**

1994

**To my parents: Lakshma Reddy and Sulochana,
my brother *late* Venugopal, and my wife and son: Sudha and Anish**

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ABSTRACT

Biofilms are a natural form of cell immobilization that result from microbial attachment to solid supports. Plastic-composite supports were prepared by high temperature extrusion of polypropylene and up to 25% (w/w) various agricultural materials and micronutrients into 2-3 mm chips. Pure cultures of the ethanol producers *Zymomonas mobilis* (ATCC 31821) or *Saccharomyces cerevisiae* (ATCC 24859), and mixed cultures with either ethanol-producing microorganism and the biofilm forming *Streptomyces viridosporus* T7A (ATCC 39115) were used. A maximum ethanol productivity of 374 g/l/h with 44% yield was obtained using soybean hull-zein-plastic composite supports with *Z. mobilis* and a 10% glucose feed. Productivities and yields were generally lower with *Z. mobilis* and *S. viridosporus* fermentations. With *S. cerevisiae*, the ethanol productivities were lower in both pure and mixed-cultures than those observed with *Z. mobilis*. Biofilm presence on the support was confirmed by weight gain, support clumping and Gram staining of supports. With these differences between pure- and mixed-culture fermentations, long-term performance studies of the pure-culture fermentations were further evaluated.

A packed bed reactor that approximated a trickling bed was custom-made for *Z. mobilis* fermentation. Soy hull-zein polypropylene composites or polypropylene supports were used. For *S. cerevisiae*, a plug-flow reactor was filled with soybean hull-soybean flour polypropylene composite supports. Glucose-yeast extract medium containing 10% glucose for *Zymomonas* and 7.5% for *Saccharomyces* was used. Continuous fermentations in replicates of two were carried out for 60 days at various dilution rates. Suspended culture fermentations were carried out in 2-liter B. Braun fermentors. There was no difference in the

productivities obtained in fermentation between the two supports for *Z. mobilis*, most likely because it is a good biofilm former. Maximum productivities of 536 g/l/h and 499 g/l/h were obtained with *Z. mobilis* using polypropylene alone and soybean hull-zein polypropylene composite supports respectively. These productivities are the highest reported in the literature so far. With *S. cerevisiae* a maximum productivity of 76.1 g/l/h was obtained on the soybean hull-soybean flour plastic composite supports.

Visible biofilm formation was observed in all the reactors within two weeks of operation. Suspension culture fermentations resulted in ethanol productivities of 4.8 g/l/h and 5.2 g/l/h with yeast and bacteria respectively. A washout was observed in suspension culture fermentation when the reactor was operated at a dilution rate of 1.0 h^{-1} . These results suggest that, biofilm bioreactors with composite support materials can be used to improve ethanol productivity and lower the costs of fermentation.

GENERAL INTRODUCTION

Ethanol, also referred to as ethyl alcohol, grain alcohol, or EtOH, is a primary alcohol with the formula C_2H_5OH . Microbial production of ethanol was an important process prior to 1940, when chemical synthesis from petrochemical feedstocks became more economical. Environmental concerns and possible future depletion of petroleum reserves has revived an interest in ethanol fermentation from renewable biomass materials such as agricultural crops and residues and wood.

Ethanol has been widely used as a solvent and extracting agent. It is useful as a fuel, in the preparation of pharmaceuticals and perfumes, and for the production of acetic acid, various lacquers, varnishes and dyes. Ethanol is denatured by the addition of a non-beverage chemical such as methanol or gasoline. Ethanol concentration is expressed in amounts of proof in which 50 and 100% ethanol is 100 and 200 proof, respectively.

Although ethanol has a wide variety of applications, the high production cost is a concern. Current ethanol production costs depend on the process used and the feedstock (42). Since ethanol is a relatively low cost, high volume product, continued sustainable growth in the ethanol industry depends on improvements in the fermentation process and recovery.

The research reported in this dissertation addresses attempts to decrease the production costs of ethanol by increasing the productivity by a biofilm reactor using yeast and bacteria. A long term study to assess the potential of these biofilm reactors for

continuous fermentation will also be discussed.

Dissertation organization

This dissertation is composed of a literature review and two papers which will be submitted to scholarly journals. The first manuscript describes the preparation of plastic composite supports and evaluation of these supports for enhanced ethanol production in biofilm reactors with pure- and mixed-culture fermentations. The second manuscript deals with a long term continuous fermentation process using the best composite supports and compares the performance of the composite supports with suspension-culture fermentations. Following the second manuscript is a general summary and conclusions. References cited in the general introduction, literature review, summary and conclusions are listed in the bibliography. The American Society for Microbiology format was used throughout the dissertation.

LITERATURE REVIEW

History

The knowledge needed to produce alcohol from grains and fruits dates back to ancient times (17). The Egyptians and Mesopotamians recorded methods to brew beer as early as 2500 B.C. Despite this ancient knowledge of converting sugar and starches to ethyl alcohol, modern techniques were not developed until after the middle of the 19th century.

The earliest studies of ethanol production through the hydrolysis of cellulose were conducted in Germany during World War I. Between 1920 and 1940 there was extensive experimentation in Europe using ethanol as a substitute fuel. The earliest recorded use of ethanol as a motor fuel is 1890. Ethanol and methanol, both monohydric alcohols, found extensive use in Europe during the pre-World War II era (76). In the U.S, alcohol fuels at that time were limited to special fuel mixtures for racing car engines. Mixtures of water and alcohol have been employed in injection into high-compression aircraft engines (17). The physico-chemical properties of ethanol are shown in Table 1.

Lignocellulose and various other carbohydrates were transformed into fossil fuels millions of years ago, thus forming the basis of our present day energy resources. Environmental concerns and possible future depletion of petroleum reserves, have revived an interest in ethanol.

Table 1. Physico-Chemical properties of ethanol

Formula	C ₂ H ₅ OH
Description	Colorless
Molecular Weight	46.07
Refractive index (at 15°C)	1.363
Boiling point °C	78.4
Flash point °C	13.9
Specific gravity	
ref to air °C/°C	20/20
ratio	0.790
Energy of combustion (KJ/L)	3.53

Applications

Two-thirds of the world's ethanol production is presently achieved by microbial fermentation. Ethanol blended with gasoline is used as a motor fuel additive and known as gasohol. Gasohol is a registered trade name for 10% agriculturally derived ethanol and 90% unleaded gasoline.

The chemical industry uses ethanol as a feedstock and as a solvent. Chemical production of ethanol is primarily from ethylene, a byproduct of petroleum refining. Ethanol can also be used to produce ethylene. Ethylene is widely used in the production of polymers like polyethylene. Ethanol can be fermented to acetic acid, which is used as a food acidulant, and as a road deicer called CMA (calcium magnesium acetate) (10). Ethanol is useful in preparations of pharmaceuticals and perfumes, for various lacquers, varnishes and dyes. It is considered appropriate for use in electric utilities as a turbine fuel for peak load requirements (95). In the U.S more

than half of the denatured alcohol produced is sold for solvent purposes in the preparations of products such as nitrocellulose coatings, shellacs, inks, hydraulic fluids, liquid detergents and soaps, deodorants, perfumes, antiseptics and lotion. Undenatured ethanol is also used in the production of vitamins, flavors and essences, mouthwashes, blood products and fortified wines (46, 47). In U.S., about 10% of the ethanol usage (excluding potable spirits) is in vinegar production (47). Of less importance is the use of ethanol as a growth substance in the production of single cell protein.

The largest market for ethanol is as an ingredient in beverages such as in liquors and alcoholic drinks. For beverage production, the raw materials consist primarily of starches or sugars. Table 2 gives an example of the various feed stocks used in the production of different alcoholic beverages.

In 1988, 400 million bushels of corn in U.S were utilized for ethanol production, adding about \$1 billion to farm income (95). In 1986, 785 million gallons of ethanol were blended into gasoline (74). Present ethanol production costs from fermentation range between \$1.15 and \$1.60 per gallon, depending on the process used and the cost of the feedstock (61). In U.S about 340 million bushels (8.6 million metric tons) of corn are used each year to produce about 850 million gallons (3.2 billion liters) of anhydrous ethanol for 10% blends with gasoline. This is enough to blend with 8% of the 112 billion gallon (424 billion liter) U.S. gasoline market (99).

Table 2. Natural feedstocks used in alcoholic beverage production

Raw material	major constituent	alcoholic product	
		fermentation	distillation
grapes	sugar	wine	brandy
apple	sugar	cider	
molasses	sugar		rum, arrack
cherries	sugar		brandy
plums	sugar	plum wine	slivovic
barley	starch	beer	corn brandy
wheat	starch	beer	corn brandy
rye	starch	beer	whisky/brandy
potatoes	starch		brandy
rice	starch	saki	arrack

Costs of ethanol production

The cost of ethanol production is less than \$1.25 per gallon depending on the process used and the feedstock costs. The cost of producing ethanol from corn starch depends on a number of factors including the cost of corn, the value of co-products, the cost of energy and enzymes, the size of the production plant, and the level of technology in the plant. The development and adoption of new technology is based on a strategy to increase the efficiency of inputs, to speed up the production process, and to raise the yield of ethanol. The development of microorganisms or strain improvement for speeding up the process, the development of markets for co-products and the development of farming technologies that raise corn yields or reduce input costs may lower the feedstock costs and save \$ 0.09 to 0.15 per gallon over the current price. Current ethanol production is reaching the theoretical limits available from the starch portion of the kernel (42). Converting the hull and other fiber portion of the kernel into

ethanol could raise ethanol yields from corn and also improve the quality of the co-product with a higher protein content. Wood (98) has discovered a bacterium that converts CO₂ and hydrogen into acetic acid. Carbon dioxide is a byproduct of ethanol production. Converting the CO₂ from the ethanol production into acetic acid is estimated to cost around \$0.75 per gallon, while the acid would sell for more than \$1.50 per gallon.

Biomass includes agricultural residues, waste streams from agricultural processing, municipal solid wastes, yard and wood wastes, and crops grown exclusively for their high energy content. Current technology for biomass conversion into ethanol is too costly for commercial applications. A kernel of corn is composed primarily of starch (70%) which is readily reduced to glucose and efficiently fermented into ethanol. Most biomass is composed of cellulose (30 to 50%), hemicellulose (25 to 35%), lignin (10 to 30%) and ash (traces), depending on the feedstock. The cellulose and hemicellulose are made up of long chains of six-carbon (glucose) and five-carbon sugars, respectively. Lignin cannot be fermented to ethanol but can be used as a combustible fuel. The pre-treatment of converting cellulose into glucose is either by harsh mineral acid treatment or by enzymes. Cellulose hydrolysis with HCl or H₂SO₄, often produces toxic byproducts and reduces some of the glucose, making it unavailable for fermentation. The alternative enzymatic hydrolysis using cellulases and hemicellulases is often too expensive. Most yeasts and bacteria are not capable of fermenting both the pentoses and the hexoses to ethanol. Ingram *et al.* (43) have genetically engineered an *Escherichia coli* that is capable of fermenting both glucose

and pentoses to ethanol.

Although ethanol has a wide variety of applications, its present high production cost is prohibitive. Raw materials are the major costs representing up to 70% of the final ethanol price (62). Therefore, a complete utilization of the raw material in fermentation is a necessity. Recovery of the product represents another important consideration in the process economics. The cost of fermentors represent a large fraction of the total equipment cost. Consequently, the use of continuous processes, which are simple to operate, which have low energy requirements, and which allow more utilization of the expensive substrate, will significantly lower the operating costs. Capital costs, on the other hand, may be reduced by using mechanically simple, small bioreactors with high rates of ethanol production.

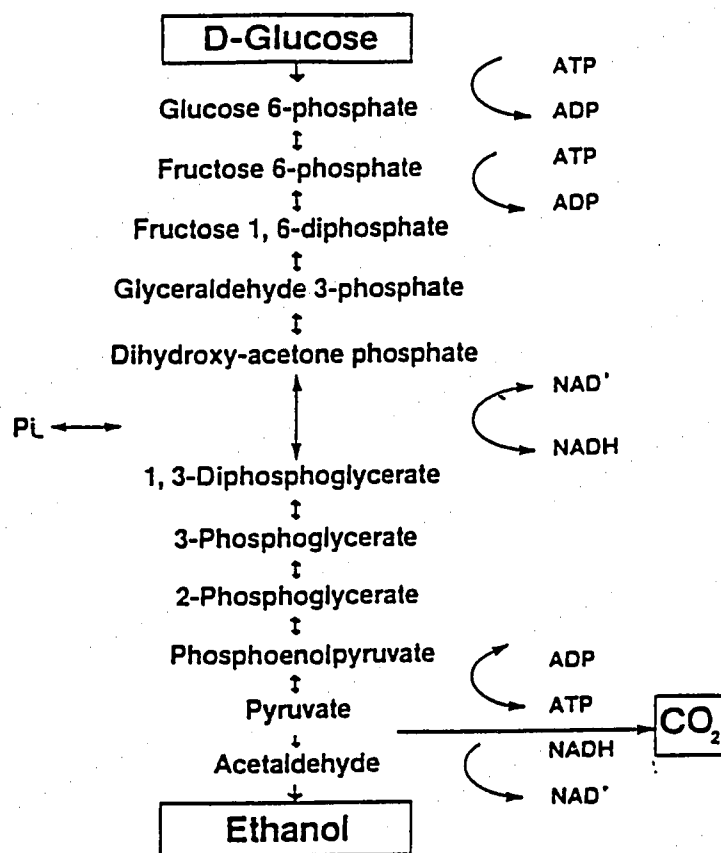
The selection of a suitable and inexpensive raw material is essential to reducing the cost of ethanol production. Since, ethanol is a low-cost, high-volume product, the economic success depends on capital and operating costs. Considerable amount of research has been carried out in the past to develop new highly efficient processes.

Microbial pathways for ethanol production

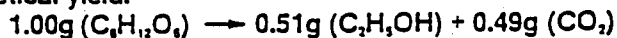
The formation of ethanol occurs by a number of well documented metabolic pathways which depend on the microorganism being employed (Table 3). For *Saccharomyces* and a number of other yeasts, ethanol is formed via the Embden-Meyerhoff-Parnas pathway (Figure 1) where, theoretically, 1 g of glucose yields 0.51 g of ethanol and 0.49 g of carbon dioxide.

Table 3. Ethanol producing Microorganisms and the major carbohydrates used as substrates (87, 92).

Organism	Substrate
<i>Saccharomyces</i> spp.	
<i>S. cerevisiae</i> and	Glucose, fructose, galactose, sucrose,
<i>S. uvarum</i> (<i>carlsbergensis</i>)	maltotriose and xylulose
<i>S. diastaticus</i>	Glucose, maltose, dextrin and starch
<i>S. rouxii</i>	Glucose, fructose, maltose and sucrose
<i>Kluyveromyces</i> spp.	
<i>K. fragilis</i> and <i>lactis</i>	Glucose, galactose, lactose
<i>K. marxianus</i>	Inulin
<i>Candida</i> spp.	
<i>C. pseudotropicalis</i>	Glucose, galactose, lactose
<i>C. tropicalis</i>	Glucose, xylose, and xylulose
<i>Pacchysolen tannophilus</i>	Glucose and xylose
<i>Schwanniomyces</i> spp.	
<i>S. alluvius</i>	Dextrin, starch
<i>S. castelli</i>	Dextrin, starch
<i>Pichia wickerhamii</i>	Xylose, cellobiose
<i>Endomycopsis fibuligera</i>	Dextrin, starch
<i>Fusarium</i> spp.	Xylose
<i>Rhizopus</i> spp.	Xylose
<i>Mucor</i> spp.	Xylose and Arabinose
<i>Monilla</i> spp.	Cellulose, xylan
<i>Zymomonas mobilis</i>	Glucose, fructose, sucrose
<i>Clostridium</i> spp.	
<i>C. thermocellum</i>	Glucose, cellobiose and cellulose
<i>C. thermohydrosulfuricum</i>	Glucose, xylose, cellobiose, sucrose, starch
<i>Thermoanaerobium brockii</i>	Glucose, sucrose, cellobiose and starch
<i>Thermobacterioides acetoethylicus</i>	Glucose, sucrose and cellobiose



Theoretical yield:



Practical yield:

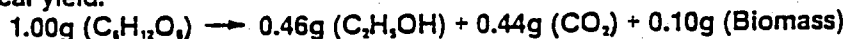


Figure 1. Formation of ethanol from glucose by the Embden-Meyerhoff-Parnas Pathway (87).

The bacterium *Zymomonas mobilis* employs the Entner-Doudroff pathway to form ethanol from glucose. Here as with the Embden-Meyerhoff-Parnas pathway, two moles of ethanol are formed from each mole of glucose utilized, but the Entner-Doudroff pathway yields only one mole of ATP per mole of glucose compared to two

moles of ATP per mole of glucose from the Embden-Meyerhoff-Parnas pathway (Figure 2). Due to interest in hemicellulose utilization, the formation of ethanol from pentoses is receiving attention. A number of yeasts, not of the genus *Saccharomyces*, can metabolize xylose through the Hetero-lactic Fermentation pathway (Figure 3) which utilizes the pentose phosphate pathway.

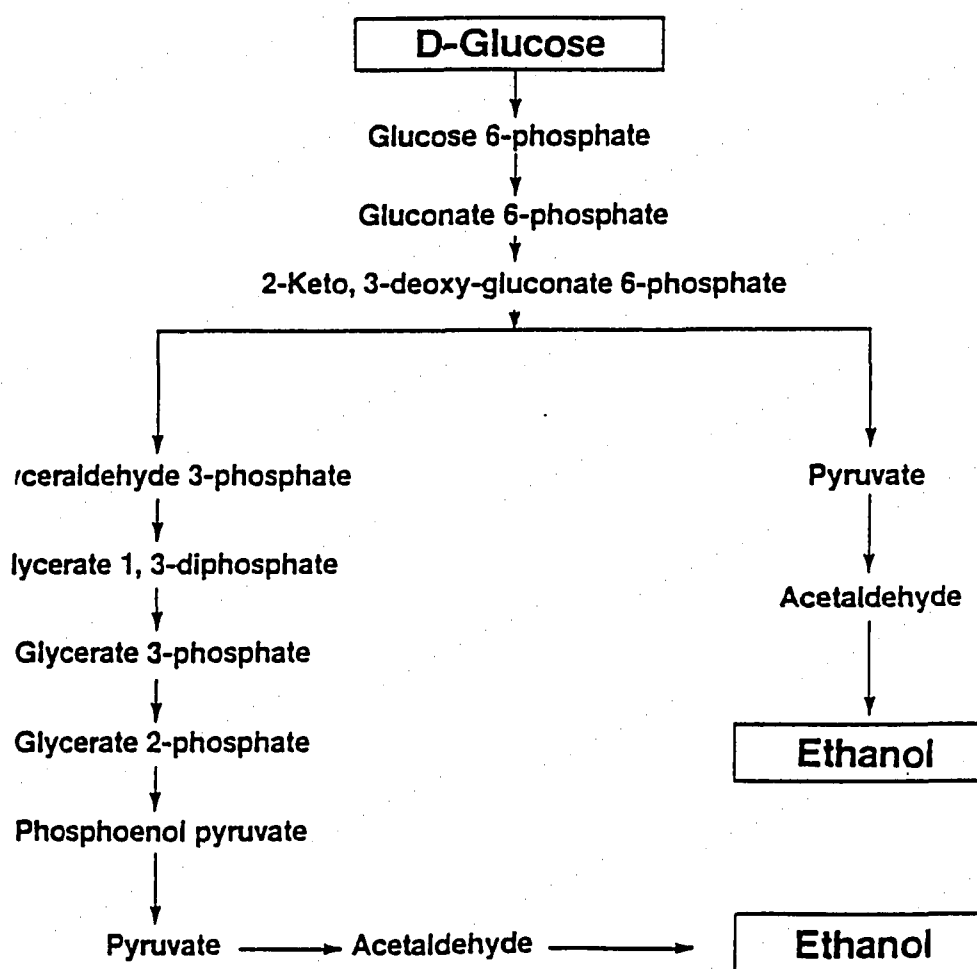


Figure 2. Formation of ethanol from glucose by the Entner-Duodoroff pathway (87).

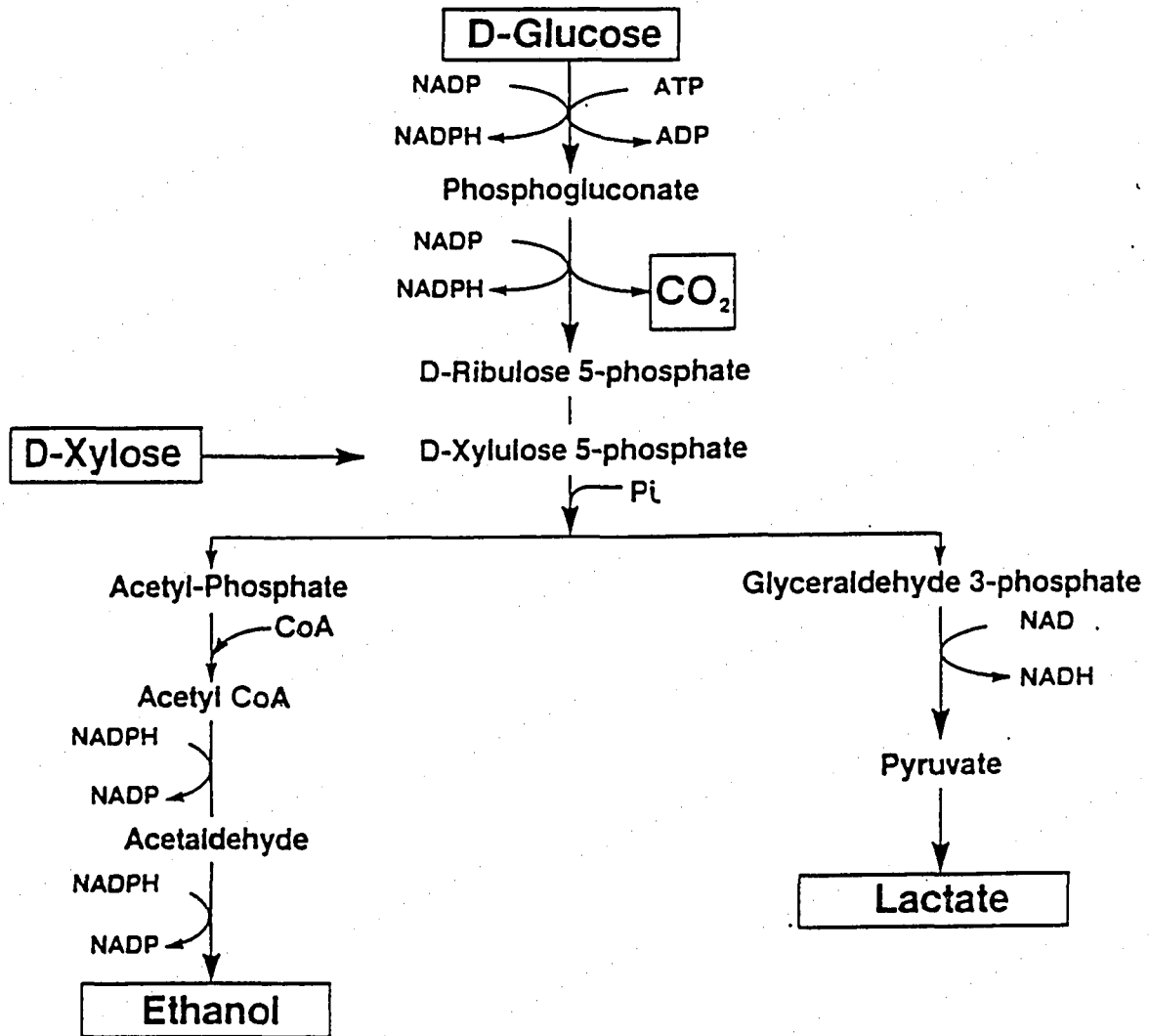


Figure 3. Formation of ethanol and lactate from glucose and xylose by the heterolactic fermentation pathway (87).

High rate bacterial fermentations

In addition to the improvements of the various process engineering parameters, research is also being directed to the use of microbes other than yeasts for ethanol production. A Gram negative bacterium *Zymomonas mobilis* has been shown to have several advantages over yeasts in the production of ethanol, with higher ethanol yields and lower biomass production than yeasts (89). Unlike yeasts, *Z. mobilis* is capable of growing under complete anaerobic conditions, so that addition of oxygen in continuous fermentation is unnecessary. *Z. mobilis* has significantly higher specific rates of glucose uptake and ethanol production when compared to yeasts (81). Techniques developed for the genetic manipulation of bacteria are being applied to improve the growth of this bacterium on sucrose, to extend the range of substrates which can be used for ethanol production, and to improve the ethanol tolerance of this bacterium (88, 25).

Feedstocks used for ethanol production

Sugars needed for the production of ethanol (glucose, sucrose, fructose) may be derived from three major classes of raw materials; sugar containing feedstocks, starchy feed materials and cellulose. Most commonly, starch derived from corn, and sucrose derived from sugarcane, are the major sources of feedstocks employed for ethanol production in the U.S. and Brazil, respectively. The use of other carbohydrate sources being evaluated for ethanol fermentation are lactose derived from whey, inulin-type poly-fructans derived from Jerusalem artichokes, and mono- and disaccharides (glucose, xylose, arabinose and cellobiose) derived from lignocellulose.

Production of ethanol from whey

Cheese whey, a major byproduct of cheese production, is highly polluting and constitutes a major waste-disposal problem for most manufacturers (20). Using free and immobilized cells of *Kluyveromyces fragilis* under batch conditions, Linko *et al.* (59) have successfully fermented demineralized whey containing 5 to 10% (w/v) lactose to ethanol in 48 h. A 90% conversion was achieved with both cell systems. Continuous production of ethanol was carried out by entrapping *K. fragilis* cells in calcium alginate beads packed in a vertical column bioreactor. At a dilution rate of 0.26 h^{-1} , and a lactose concentration of 5% (w/v), an effluent ethanol concentration of 2% (w/v) could be maintained for at least 31 days. However, at higher lactose levels the efficiency of the bioreactor declined due to the inability of the *K. fragilis* strain to tolerate high levels of ethanol.

Since relatively few yeasts are capable of directly fermenting lactose to ethanol, the co-immobilization of β -galactosidase enzyme with *S. cerevisiae* cells has been attempted. Hahn-Hagerdal (38) covalently attached β -galactosidase to alginate. The alginate-enzyme complex was then co-entrapped with yeast in calcium alginate beads. Using a vertical packed-bed bioreactor, and a 4.5% (w/v) lactose feed in whey permeate, an ethanol concentration of up to 1.5% (w/v) was achieved for at least 20 days.

Production of ethanol from Jerusalem artichokes

Growing energy crops on marginal land as an additional feedstock for ethanol production appears promising, as it will not threaten food supplies. One such carbohydrate-rich plant is Jerusalem artichoke (*Helianthus tuberosus*). This has a low fertilizer requirement, grows well on poor secondary land and is resistant to frost and plant diseases. *Kluveromyces fragilis* cells entrapped in calcium alginate beads were used for the repeated batch production of ethanol from extracts derived from Jerusalem artichokes. Margaritis and Bajpai (68) studied the continuous production of ethanol from Jerusalem artichoke extracts in a vertical packed-bed bioreactor containing alginate entrapped cells of *K. marxianus*. A maximum ethanol productivity of 56.8 g/l/h was achieved with an ethanol concentration of 65.3 g/l by utilizing 83% of the available sugars.

Production of ethanol from sweet sorghum

Sweet sorghum (*Sorghum bicolor*) has the potential of becoming a useful energy crop. The primary advantages of sweet sorghum are its adaptability to diverse climatic and soil conditions and its reduced need for nitrogen fertilizer and water when compared to more conventional crops such as corn (60). From the agronomic aspect, the use of crop rotation has been known to increase crop yields. Three-year crop rotation, such as corn-soybean-wheat has shown to produce a better soybean yield. If the crop of the rotation were a non-grain crop such as sweet sorghum, world prices of grains, particularly corn, would increase, adding income to farmers. Despite the advantages,

sweet sorghum is yet to become a viable alternative to corn in fuel ethanol production. This is because most processes are based on separating and then fermenting the sugar fraction of the stalk. These processes are uneconomical, energy inefficient, or unproven on a commercial scale (32).

Production of ethanol from cellulose and cellobiose

Cellulose is the most abundant organic compound in the biosphere. It is a major constituent of plant material and is constantly being replenished by photosynthesis. The enzymatic saccharification and alcohol production from cellulose has been extensively studied. The enzymatic hydrolysis of cellulose is affected by the synergistic action of exo-cellulase, endo-cellulase and β -glucosidase. Cellobiose and glucose, the end products of cellulose saccharification inhibit the enzymic hydrolysis of cellulose. Attempts have been made to overcome the end-product inhibition of the cellulolytic enzymes by simultaneous saccharification and fermentation. Hagerdahl and Maosbach (38) devised a procedure for the continuous production of ethanol from cellobiose by using β -glucosidase co-immobilized with baker's yeast cells. β -Glucosidase from sweet almonds was first covalently bound to the carboxy groups of sodium alginate. The alginate β -glucosidase complex was then mixed with the cells of *S. cerevisiae* and the mixture precipitated by calcium ions in the form of calcium alginate beads. These beads were then packed in small vertical column and cellobiose was fed continuously. The theoretical yield was reached after two days of operation but after two weeks of operation the system had only 10% of the maximum activity.

Immobilized cell systems

Historically, microbial cells attached to surfaces have been exploited to perform several functions. Waste-water treatment, leaching of mineral ores, and production of high fructose corn syrups are but a few examples of the existing processes (21). The production of ethanol from glucose, requires the sequential action of nine different enzymes in addition to the two coenzyme pairs, ATP/ADP and NADP/NAD. In order to achieve such a conversion, the constant regeneration of the coenzymes is essential. This can only be achieved if the immobilized cell is maintained in the viable state. Thus, although several techniques have been developed for the immobilization of cells, not all of these can be readily applied to fermentation systems requiring viable cells. It is therefore essential that any technique developed for immobilization of live cells must be mild enough to retain the viability of cells (55).

Definition of immobilized cell systems

Abbot (1), defined immobilized cells as any system in which microbial cells are confined within the bioreactor, thereby permitting their economical reuse. The term economical has been used to exclude processes in which cells are recovered and reused by employing techniques such as centrifugation and microfiltration. The following techniques of live cell immobilization have been used in ethanol production:

1. Mechanical containment of cells in a bioreactor.
2. Cell attachment to solid supports by electrostatic, ionic or covalent interactions.
3. Physical entrapment of cells within polymeric matrices.

4. Immobilization without inert supports (cell flocculation).

Criteria for selecting a cell immobilization method

In selecting a suitable technique for live cell immobilization, the following criteria must be considered (64):

1. The method of immobilization is mild enough to ensure cofactor regeneration capability.
2. The process is capable of regeneration following deactivation which may occur after long-term operation.
3. The immobilization technique is such that high biomass concentrations can be achieved within the bioreactor and retained at that level for an extended period of time.
4. The immobilization method is simple and inexpensive.
5. The immobilized cell is stable at the operating pH and temperature.

Advantages of immobilized cell systems

Immobilized cell systems confer desirable properties to a biological process which are not achieved in conventional batch and continuous fermentation systems. Although the advantages depend specifically on the method of immobilization, some generalizations can be made. These include:

1. High cell concentrations and therefore higher reaction rates.
2. Higher dilution rates are achieved without cell washout.

3. Less susceptible to the effects of inhibitory compounds.
4. The combined effect of high cell density and operation at high dilution rates reduce risk of reactor shut-down due to microbial contamination.

The important characteristics of an immobilized cell system and/or support are listed in Table 4.

Ethanol production using immobilized cell systems

The continuous production of ethanol was first described by Delbruck in 1892 (29) and subsequently by Barbaret in 1899 (7). These systems were, however, impractical owing to their primitive vessel design and construction, and the failure to guard these processes against microbial contamination. Nevertheless, these early methods established the fact that the maintenance of high cell densities was essential to achieve rapid fermentation (78). In most cases, the advantages and limitations of a given bioreactor system depend on the method of immobilization and the reactor configuration used.

Ethanol production using mechanically-contained cell systems

In 1892, Delbruck (29) described a system employing a porous cylinder within which a yeast was maintained. The use of such a filter was intended to restrict the loss of cells from the vessel, enabling the accumulation of yeast cells to a high concentration, in order to achieve rapid fermentation.

Table 4. Characteristics of immobilized cell systems (65)

A.	Immobilization method
	1. Entrapment within matrices
	2. Attachment to solid support by adsorption or covalent bonding
	4. Microencapsulation within polymeric membranes
	5. Immobilization without inert supports (flocculation)
B.	Microbiological characteristics
	1. Cell viability and function
	2. Cell growth rate and yield
	3. Metabolic products and yields
	4. Cell wall and membrane permeability
	5. Cell concentration
	6. Respiration requirements
C.	Chemical characteristics of the matrix
	1. Chemical composition and method of synthesis
	2. Functional groups, monomers types
	3. Possible toxicity to cell function and viability
D.	Physical characteristics of the matrix
	1. pH and temperature stability
	2. Solubility characteristics in aqueous solutions
	3. Porosity characteristics
	4. Oxygen transfer characteristics for aerobic system
	5. Geometry and size of the matrix
	6. Mechanical strength of the matrix
	7. Specific gravity and fluidization velocity
E.	Stability characteristics of the cell matrix system
	1. Activity and half-life in continuous operation
	2. Operational stability in a given bioreactor system
	3. Possible stabilization of the cell enzymatic system
	4. Stability and activity preservation during storage

Plug-flow fermentors consisting of a mixture of yeast and kieselguhr (Celite 545) held between two filters were used for the production of beer (6). The plug-flow fermentors can operate at fermentation rates several times higher than the simple batch systems. They do, however, suffer from plugging problems, and the need to constantly

regenerate the yeast cells under aerobic conditions, which will interrupt sustained industrial operation. Furthermore, high pressure is needed to force the medium through the system.

High cell densities may also be maintained within dialysis bioreactors. This bioreactor uses a dialysis membrane which separates the fermentation zone from the nutrient reservoir. Thus, the nutrients diffuse through the dialysis membrane into the fermentation zone and the product diffuses back into the nutrient zone, where it is recovered in an overflow (82). A limitation of the simple continuous dialysis bioreactor is the gradual fouling of the membrane and the inherently slow process of diffusion through the limited surface area of the dialysis membrane. These problems have been overcome by pressure dialysis, as in the Rotorfermentor (66). In a small scale pilot plant of the Rotorfermentor employing cells of *S. cerevisiae* ATCC 4126, almost complete utilization of glucose was possible at a feed concentration of 104 g/l. Although the Rotorfermentor appears to be attractive in terms of productivity, it is mechanically complex and difficult to operate. If periodic replacement of the membrane is necessary, then this will interrupt the continuous production when operating on an industrial scale.

Cell densities of 155 g/l and productivities of 70 g/l/h have been reported when fermenting glucose to ethanol. Hollow fiber bioreactors, however, are complex and costly. Carbon dioxide venting and membrane plugging may be encountered. These reactors, therefore, may not be important for the industrial production of ethanol (67).

Ethanol production using attached cell systems

The attachment of microbial cells onto solid supports may be accomplished by two different methods:

1. Attachment of cells to a support by electrostatic interactions.
2. Attachment of cells to a support by either covalent bonding or by using cross-linking agents.

Adsorption of cells to solid supports

Any surface in contact with a nutrient medium which contains suspended microorganisms will, in time, become biologically active due to cell adhesion. This innate ability of most microorganisms has been exploited as a useful cell immobilization technique (5). Although the mechanisms of these support-cell interactions are not fully elucidated, it is believed that they occur as a result of the charged nature of the microbial cell wall.

When operating at high dilution rates using an adsorbed cell system, the resulting high fluid flow rates may very easily detach cells from the solid support, causing washout. (72). It is therefore desirable that in addition to retaining a high cell density, the support material must have a high affinity for the microbial cells.

Moo-Young *et al.* (70) reported that yeast cells immobilized on wooden chips had 100% retention of cells even after the vertical packed-bed reactor was operated continuously for 30 days. Gencer and Muthurasan (34) also reported high retention of yeast cells by wood chips.

Experiments with *S. cerevisiae* have shown that macroporous material adsorbs the organism more than the non porous borosilicate glass (69). These workers have also studied the effect of various pore diameters on cell accumulation. They observed that to allow the passage and immobilization of yeast, the pore size must be approximately four times the maximum diameter of the yeast cell employed.

A variety of supports have been utilized for the adsorption of yeast and bacterial cells for the continuous production of ethanol. Organic supports such as carrageenan gels (45), alginate in a three-stage reactor (52), ion exchange resins (53) and calcium alginate (2) were used for fuel-grade ethanol production. Inorganic supports such as vermiculite (9), and γ -alumina (54) were also used for immobilization of cells for ethanol production. Use of cells immobilized in solid gel matrices as beads has been studied in packed beds and in fluidized bed reactors (99).

Ghose and Bandyopadhyay (35) reported that the ethanol productivity with an immobilized cell bioreactor was seven times higher than that obtained in a free cell system. Tyagi and Ghose (93) adsorbed cells of *S. cerevisiae* NRRL-Y-132 on to an unspecified inert support and used cane molasses as feed. The reducing sugars content was 150 g/l when operated at a dilution rate of 0.375 h^{-1} . The system achieved an ethanol productivity of 28.6 g/l/h with 96% of the sugars being utilized. Ethanol productivities as high as 132 g/l/h were reported with *Z. mobilis* cells adsorbed to circular disks of borosilicate glass-fiber-pads packed in a vertical column bioreactor (3, 4). However, vertical packed bioreactors are associated with different levels of CO_2 gas hold-up which results in poor heat and mass transfer properties, reduced bioreactor

efficiencies and high pressure drops along the length of the bioreactor. In order to alleviate these problems, fluidized and horizontal column bioreactors have been employed (64).

Bland and co-workers (9) used an attached film expanded-bed bioreactor to facilitate mass transfer and reduce CO₂ hold-up in the bioreactor. Cells of *Z. mobilis* were adsorbed onto vermiculite particles. A maximum ethanol productivity of 105 g/l/h was achieved at a dilution rate of 3.6 h⁻¹. Under these conditions, only 64.2% of the substrate was consumed. A period of 24 h was required to achieve a steady-state condition and adequate adsorption of the cells to the support particles. An attached film expanded bed (AFEB) bioreactor has also been utilized for the production of ethanol from cheese whey using adsorbed cells of *Saccharomyces fragi*.

The adsorbed cell system is limited by two major constraints. First, the amount of biomass that can be adsorbed by a unit gram of the carrier is limited by the surface area of the support particle. Second, the operational stability of the bioreactor system is restricted by the rate of desorption of cells from the support. This problem is especially severe when changes in pH or ionic strength occur, or when cells are sheared from the carrier surface by virtue of rapid flow and high turbulence created by CO₂ bubbles.

Attachment by covalent bonding and cross-linking agents

This method of immobilization is based on a covalent bond formation between an activated support and cells. This requires the use of a cross-linking agent. However, due to the toxicity of most of the reagents used, covalent binding of cells to a carrier is

not frequently employed. The preservation of the cell viability is of utmost importance in ethanol fermentation. This technique, however, does have the advantage that the cells are linked to a uniform surface by bonds which are stable for long periods, so that cell leakage from the bioreactor is minimized.

It is well known that cell membranes of microorganisms consist of polysaccharides, protein-lipid complexes and teichoic acids. Glutaraldehyde, a common cross-linking agent, has been known to react readily with the protein present in the lipid-bilayer of cell membranes. By adsorbing gelatin to an inert support, a reactive base for glutaraldehyde is provided. This allows a covalent link to be formed between the microbial cells and the gelatin support. Sitton *et al.* (82) employed a method where gelatin (25% w/v) coated Raschig rings were sprayed with a 3% (w/v) glutaraldehyde solution and dried for 24 h. Actively growing cells of *S. cerevisiae* were attached to the gelatin coated rings by circulating a cell suspension through a vertical packed bed column. Severe channelling due to CO₂ hold up and cell overgrowth occurred.

Ethanol production using entrapped cell systems

Cell immobilization by entrapment occurs with the inclusion of cells within a rigid polymeric matrix. In immobilization techniques, entrapment includes both enclosure of a catalyst behind a membrane and within a gel structure. Carrier binding includes all methods where there is a direct binding of cells to water-insoluble carriers by physical adsorption or by ionic and/or covalent bonds. Potential mass transfer limitations are always present with an entrapment system, either across the gel matrix or

gel occlusion, or across the system membrane in membrane reactors. On the other hand, the carrier binding method allows direct contact between the fermentation medium and the biocatalyst, and the medium flows in and out of the system without restriction, thus minimizing mass transfer problems. In gel entrapment system the most active cells are at the gel surface, and agitation of the beads leads to loss of activity due to leakage of the outer layer. Gel entrapment systems often require a continuous supply of chemicals to maintain the hardness of the beads.

Alginate entrapped cell systems

Alginate is a glycuronan consisting of residues of D-mannuronic acid and L-glucuronic acid arranged in a blockwise fashion along a polymer chain (41). In the presence of multivalent cations gel formation occurs. The immobilization is attractive primarily because of its simplicity. It is achieved by dropping a mixture of sodium alginate solution and cell suspension into a calcium chloride solution. Furthermore, the immobilization reagents are of low cost, making the procedure feasible for large scale applications. The porosity and diffusion properties of sodium alginate pellets have been known to be influenced by the concentration and type of sodium alginate and calcium chloride used (12, 50).

In order to facilitate the retrieval of immobilized cells from batch and fed batch systems, the use of magnetically immobilized cells has been employed by Birnbaum and coworkers (8). They observed that small beads (1-mm diameter) were more metabolically efficient than larger beads (3-mm diameter), indicating that the mass

transfer limitation within larger beads reduced the fermentation rate.

Cho *et al.* (18) compared the performance of a fluidized bed bioreactor and a vertical packed bed bioreactor, both employing alginate-entrapped yeast cells as a biocatalyst. Using a 10% (w/v) glucose feed, the ethanol productivity of a fluidized-bed bioreactor (FBBR) was 21.2 g/l/h, and that of the vertical packed-bed bioreactor was 10.0 g/l/h. The superior performance of the FBBR was attributed to the absence of CO₂ hold-up.

McGhee (71) investigated the production of ethanol from glucose by calcium alginate entrapped yeast cells that were 24, 48, 72 and 96 h cultures. They observed that the older yeast cells were much more efficient than the younger cells.

A volumetric productivity of 14.90 to 17.41 g/l/h with 87 to 97% conversion was obtained in a multistage fluidized-bed bioreactor. This bioreactor alleviated the problems associated with CO₂ evolution. Tzeng and Fan (94) reported a specific ethanol productivity of 18.37 g/l/h by using immobilized yeast cells in a multistage fluidized-bed reactor.

A major disadvantage of calcium alginate as an immobilization support is that moderate concentrations of calcium chelating agents and certain cations such as phosphates, EDTA, Mg⁺² and K⁺ disrupt the gel by solubilizing the calcium (12). Several other materials for improving the physical and chemical stability of the calcium alginate gel have been described. The use of divalent cations other than calcium as gel inducing agents has been suggested. Paul and Vignais (76) demonstrated that Sr²⁺ and Ba²⁺ produced gels of greater mechanical and chemical stability than Ca²⁺.

κ -Carrageenan entrapped cell systems

κ -Carrageenan is a polysaccharide isolated from seaweeds and is composed of unit structures of β -D-galactose sulfate and 3,6-anhydro- α -D-galactose. κ -Carrageenan can be easily induced into gel formation by contact with a solution containing a number of gel-inducing agents such as metal ions, amines and water-miscible organic solvents. Greater stability has been achieved by further treatment with hardening agents such as glutaraldehyde, polyacrylamide and hexamethylenediamine (91).

The growth of a *Saccharomyces* spp. entrapped in κ -carrageenan has been studied by Wada and co-workers (96). They observed that the immobilized growing cell system had enlarged colonies assembled near the gel surface upon incubation in a nutrient medium. Using a horizontally packed-bed bioreactor with cells of *Z.mobilis* immobilized in κ -Carrageenan, an ethanol productivity of 101 g/l/h has been achieved with a glucose concentration of 108 g/l (65) .

Entrapment of cells within other polymeric matrices

Cell entrapment in polyacrylamide involves the polymerization of an aqueous solution of acrylamide monomers in which microorganisms are suspended. The porosity of the gel is a function of the degree of cross-linking, which in turn depends on the relative amounts of the acrylamide monomer and the bi-functional cross-linking agent used (44). Due to the toxicity of the acrylamide monomer, the temperature used for the polymerization process, the duration of cell contact with the monomers, and the time required for gel formation, all dictate whether the cell viability is retained or not. Siess

and Divies (85) observed that the polymerization process may destroy 40 to 80% of the cells depending on the physiological state. The production of ethanol using cells of *S. cerevisiae* entrapped in polyacrylamide gel has been studied by Shiotani and Yamane (84).

The enzyme activity and cellular integrity may be readily impaired due to the denaturing effects of polyacrylamide. Polymerization is also hindered when high biomass loading is used. Polyacrylamide gel pellets are usually of irregular shapes and sizes and in columns these pack irregularly, causing uneven flow and the development of relatively high, flow-induced pressure drops.

Limitations of entrapped cell systems

Entrapped cell systems are subjected to mass transfer limitations imposed by the diffusion barrier created by the support matrix. High levels of ethanol may accumulate within the gel and therefore reduce the efficiency of the system. In ethanol fermentation systems, CO₂ is also a major product. The gas produced has relatively low solubility in aqueous media and therefore the diffusion of CO₂ out of the matrix is rate limiting. The accumulation of the gas in the matrix causes gel disruption, and also adversely affects the metabolism of the entrapped cells (64). Table 5 lists a summary of immobilized cell used for ethanol fermentation.

Table 5. Summary of immobilized cell ethanol fermentation

Microorganism	Substrate	Ethanol Conc./ Productivity	Type of reactor/ Special technique	Ref
<i>Saccharomyces cerevisiae</i>	Glucose	125 g/l after 5 h	Hollow porous alumina containing gel immobilized cells	93
<i>Saccharomyces cerevisiae</i>	Sweet sorghum juice	7.3-9.7 % yield	Sugar conversion efficiency reached up to 90%	63
<i>Saccharomyces cerevisiae</i>	Sugar cane juice	135 g/l in 8 h	On line removal of toxic end products by high alcohols & activated carbon	99
<i>Saccharomyces cerevisiae</i>	Sugar cane water suspension	42-53 g/l	Simultaneous extraction and fermentation	81
<i>Saccharomyces uvarum</i>	Non aseptic cane molasses	6.2 g/l/h	Continuous stirred tank reactor with five stage system for substrate recirculation	13
<i>Zymomonas mobilis</i>	Glucose	13 g/l/h	Batch vertical rotating immobilized cell reactor	2
<i>Zymomonas mobilis</i>	Glucose	63 g/l/h	Continuous vertical rotating immobilized cell reactor	2
<i>Zymomonas mobilis</i>	Glucose	42-46 g/l/h	Cell reactor separators with trickle flow operation and sponge as packing	57
<i>Zymomonas mobilis</i>	Sucrose	92 g/l/h	Sugar conversion efficiency of 60% with 10% sucrose feed. Culture isolated from sugarcane juice	80

Ethanol production using flocculated cell systems

The flocculant nature of certain yeasts and *Z. mobilis* cells has been used as a means of maintaining a high cell population within a bioreactor without the need for added materials to support film growth and retain cells (1, 13, 31).

Various continuous fermentation bioreactor configurations have been applied for production of ethanol using flocculant cells. These include stirred-tank fermentors, slant tubes, vertical packed columns and tower fermentors. However all these have some features that restrict their application. They can be operated only within a narrow range of dilution rates to avoid cell washout. Reactor packing can inhibit flow. Floc break and flotation of cells occurs due to gas release.

Types of bioreactors used for ethanol fermentation

Batch fermentation

In 1984, up to 75% of ethanol from fermentation was produced in batch processes. In this process, a large volume of the substrate (10 to 15% [w/v] of sugar supplemented with nutrients) is fed to a fermentor and inoculated with a 5 to 10% inoculum of actively growing yeast. Yeast growth and alcohol production are allowed to proceed until maximum yields are obtained. At this point, the reaction mixture is placed into a holding tank, the fermentor cleaned and sterilized and a new batch started. Thus, in a batch fermentation, the lag and log growth phases of the yeast represent a major drawback to more rapid fermentation, and the downtime associated with the

emptying, cleaning and refilling of the fermentor also contribute to the overall low ethanol productivity.

Batch fermentor

The batch fermentor consists of a closed vessel, flask or a continuously stirred tank reactor (CSTR) containing a suitable growth medium and operated under optimum conditions of temperature, pH and dissolved oxygen. The reactor is inoculated with a starter culture and the cells allowed to grow until some essential component of the medium is exhausted or the environment changes due to the accumulation of a toxic product or change in pH. The reactor is equipped with a pH controller, foam controller, agitation and gas sparging devices (Figure. 4). The production of an end product and the changes caused in the environment of the fermentor limits the growth of the microorganisms. Therefore, continual removal of the end product and addition of new medium to the fermentor will improve product formation rates, improve yields, and decrease the capital and operating costs. In a batch fermentation, cell growth and ethanol production is continued until maximum yields have been obtained.

Continuous fermentation

Substantial improvements over traditional batch processes result when the fermentation is made continuous. Continuous systems, with their attendant preparation and product separation equipment, are generally smaller than those used in equivalent batch systems, and therefore, result in lower capital costs.

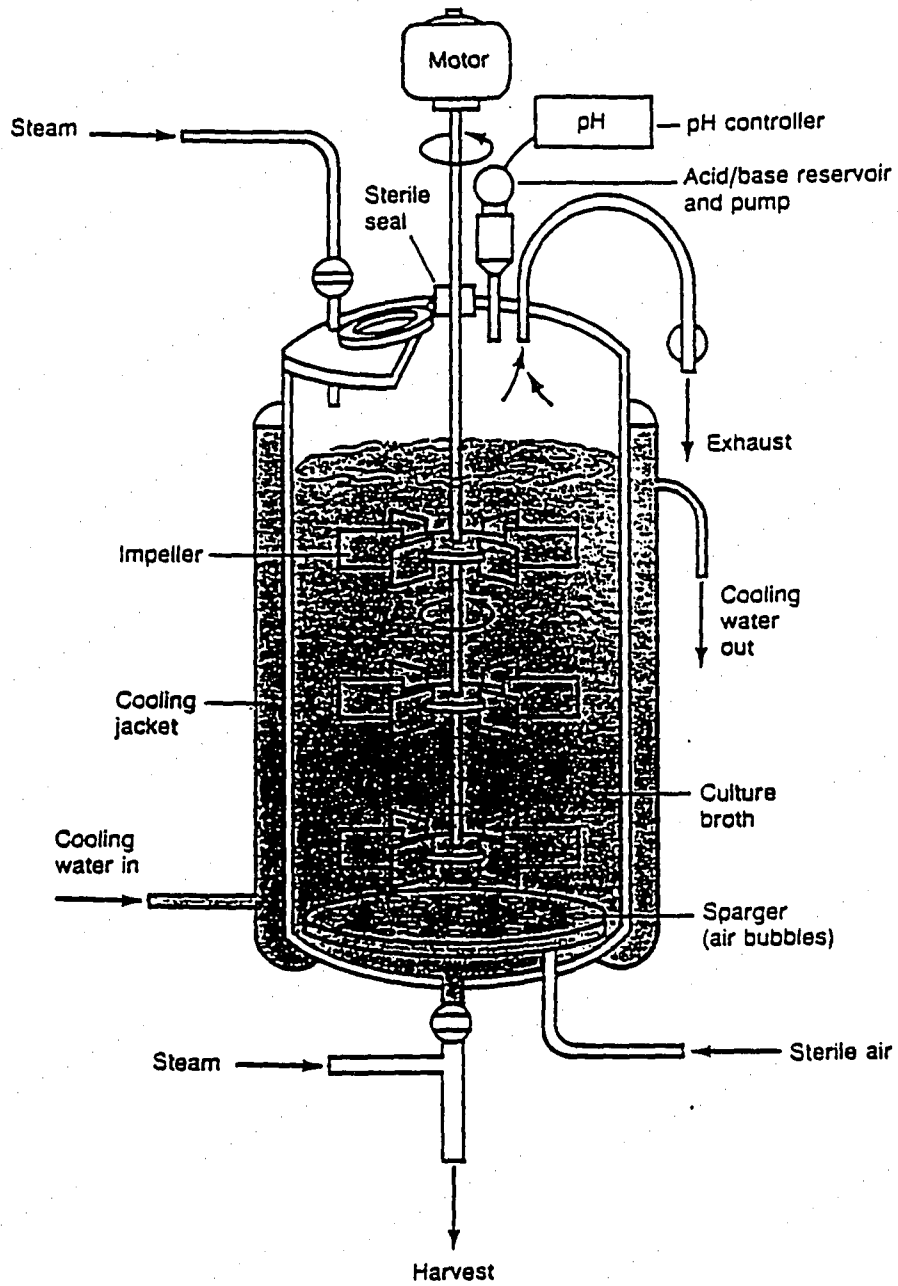


Figure 4. Schematic diagram of a batch type fermentor (21).

In a continuous fermentor the microbial population is maintained in a continuous state of balanced growth by continuously removing some of the culture medium and cells and replacing them with a fresh medium at the same rate. There are two types of continuous culture systems:

1. the chemostat, which operates by supplying an essential growth-limiting nutrient at a constant rate with the bioreactor working volume being controlled by a spill-over spout,
2. the turbidostat, which is operated by maintaining a constant cell density by constantly measuring the optical density and by supplying fresh medium as required. The bioreactor volume is kept constant by placing the whole reactor on load cells and by maintaining a constant reactor weight by controlling the outlet pumping rate.

All these systems require a method for controlling a constant bioreactor volume. This can be done by using an overflow tube at a constant height within the bioreactor vessel, so that as fresh medium is pumped into the bioreactor an equal volume of the culture enters an overflow tube and passes to the collection vessel. It is also possible to have a pump connected to the culture outlet, carefully maintaining the pumping rate equal to the medium inlet flow rate. These reactors are equipped with temperature, Ph and dissolved oxygen controllers. Higher productivities can be achieved by continuous fermentation, but cell wash-out may occur at high dilution rate. The dilution rate is defined as the volumetric flow rate (in and out) divided by the fermentor volume.

Higher ethanol productivities can be achieved by employing a high concentration of yeast or bacterial cells within the bioreactor, with continuous removal of end product. The system needs to be well-mixed to avoid short-circuiting of feed between inlet and outlet. Therefore, continuously stirred tank reactors are required.

Cysewski and Wilke (23), using an adapted yeast culture, achieved a cell density double that obtained in a batch culture and an ethanol productivity of 6 g/l/h, which is about three times higher than that obtained in the batch culture.

Two-stage continuous-flow bioreactor

The performance of a continuous fermentation, however, is limited by ethanol inhibition. In order to obtain higher ethanol concentrations with reduced product inhibition, multistage continuous fermentation is employed. Ghose and Tyagi (36), obtained final ethanol concentrations of 80 g/l using a two-stage CSTR with ethanol productivities two to three times greater than that of a single-stage continuous flow CSTR. The CSTR does, however, have definite productivity limitations which depend on the growth characteristics of the organism. For instance, in a single-stage continuous flow bioreactor at steady state, the specific growth rate (μ) equals the dilution rate of the system. At dilution rates exceeding the maximum specific growth rate (μ_{\max}), the culture washes out of the fermentor.

A two-stage, continuous-flow bioreactor, containing both immobilized and suspended free yeast cells was developed by Gil *et al.* (33) (Figure. 5). A ceramic-like matrix material was used as the support for cell attachment or biofilm formation. The

immobilization was performed by circulating the yeast slurry through the support material for 10 h. Using a synthetic sugar cane juice supplemented with mineral salts, yeast extract, peptone and aeration combined with a liquid recycle ratio of 20:1 within each reactor stage, the highest ethanol productivity of 28.5 g/l/h was obtained at a dilution rate of 0.25 h⁻¹ with 82% of the sugar consumed. This system was operated for two years. High test molasses (commercially concentrated, natural sugarcane juice) was an acceptable feedstock only when supplemented with ammonium salts.

The performance of continuous fermentation is limited by ethanol inhibition. In order to obtain higher ethanol concentrations with reduced product inhibition, the use of CSTRS arranged in series has been suggested. An ethanol concentration of 80 g/l was obtained by using a two-stage CSTR with ethanol productivities two to three times greater than that of a single-stage CSTR.

Multi-stage continuous stirred tank reactor

In a single-stage CSTR the high ethanol concentration in the reactor reduced the rates of yeast growth and product formation. A multi-stage, multi-feeding CSTR system for continuous fermentation of cane molasses to ethanol was developed by Chen (14). The productivities obtained from a three-stage (5.4 g/l/h) and a five-stage (6.2 g/l/h) system were significantly higher than from a single-stage (4.6 g/l/h) system. When the five-stage system was scaled up to a 1,000 times in a pilot-plant, the effluent ethanol concentration and overall volumetric productivity were 8.5% (v/v) and 5.3 g/l/h, respectively. This system (Figure. 6) was operated for fermentation of non-aseptic cane molasses.

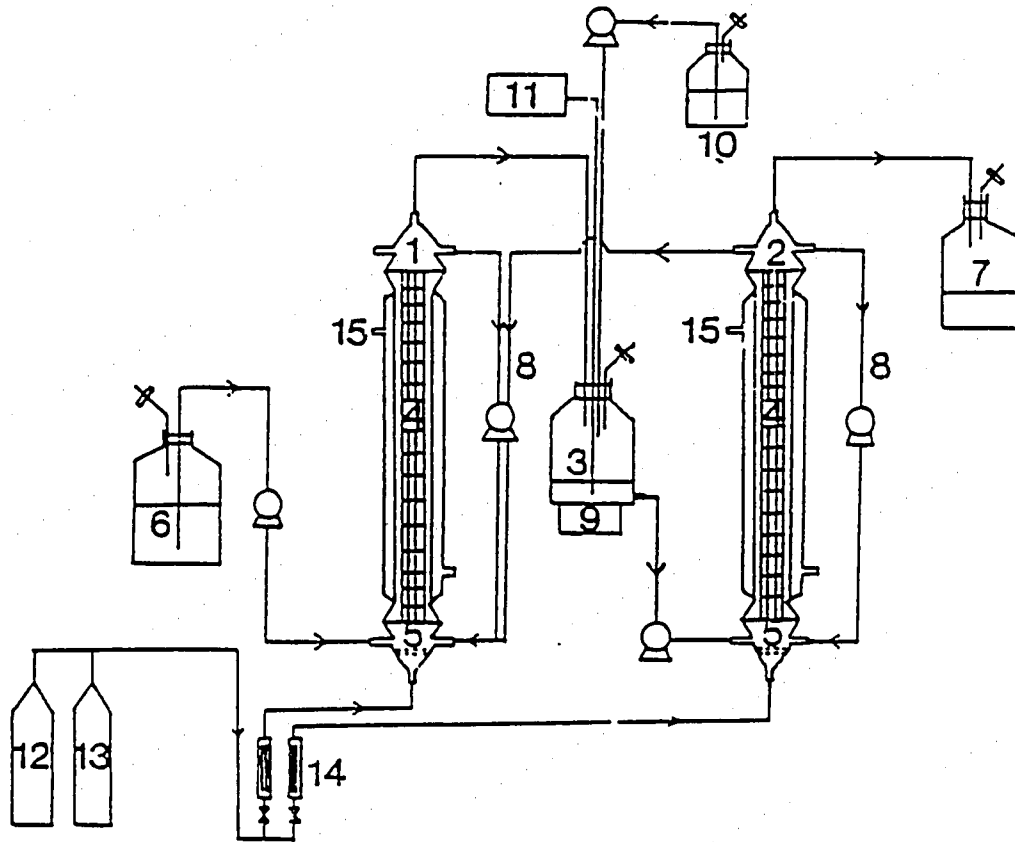


Figure 5. Schematic diagram of two-stage immobilized cell bioreactor for continuous ethanol production (53).

- | | | |
|--------------------------|--------------------------|-------------------------|
| 1. Reactor stage one | 2. Reactor stage two | 3. Surge tank |
| 4. Immobilization matrix | 5. Sintered glass filter | 6. Sugar feed reservoir |
| 7. Effluent reservoir | 8. Recycle line | 9. Magnetic stirrer |
| 10. Nutrient reservoir | 11. pH meter | 12. Air cylinder |
| 13. Nitrogen cylinder | 14. Gas flow meter | 15. Water jacket |

Continuous fermentation with cell recycle

Continuously stirred tank reactors with recycle

In this system, cells released in the product stream are collected by centrifugation or filtration and reintroduced into the fermentor. This permits the fermentor to operate at higher dilution rates and, therefore, increases ethanol productivities. The use of the centrifuge for cell separation does, however, increase the capital cost, requires considerable maintenance, added supervision, elevated energy requirements, and is associated with an increased risk of contamination. Ethanol productivities of 30-40 g/l/h have been obtained (23).

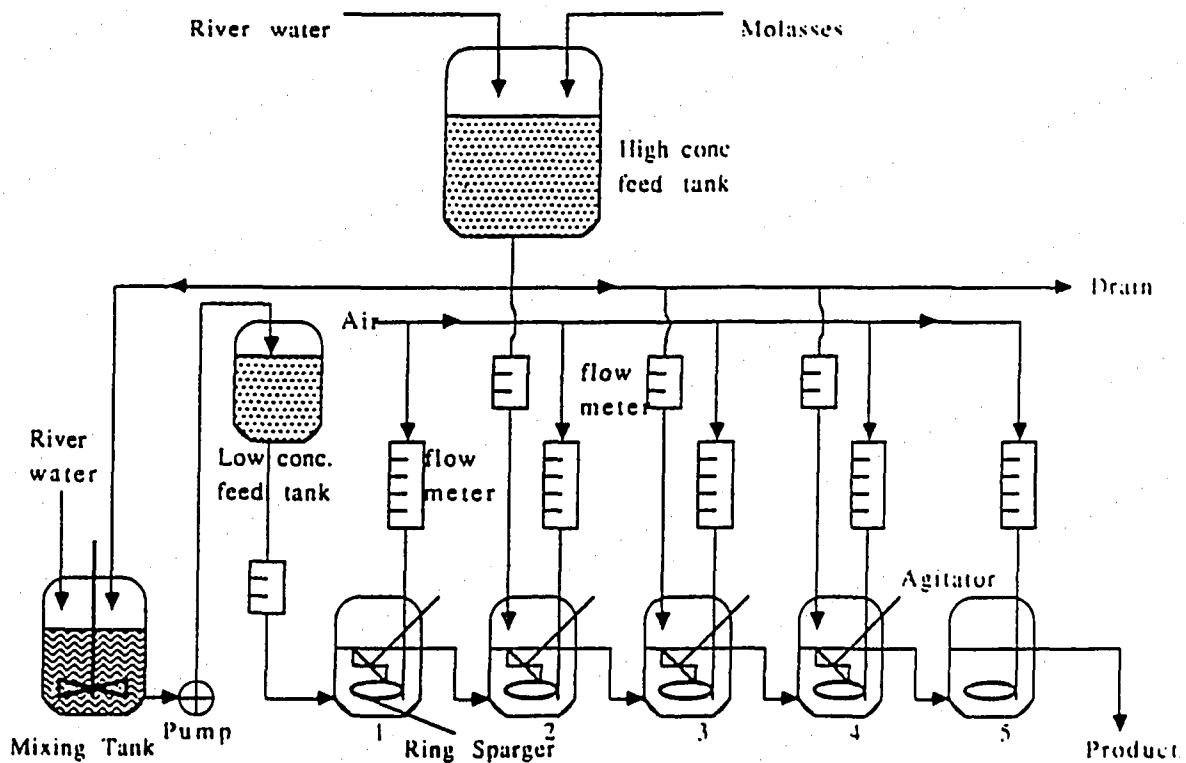


Figure 6. Schemtaic diagram of a multistage CSTR (14).

Membrane recycle fermentor

A membrane recycle bioreactor for ethanol production from sulfuric acid treated whey permeate was designed by Tin and Mawson (90) (Figure 7). The fermentation broth was recycled through a ceraflo ceramic membrane filter (MF) (pore size 0.45, 25 μm and total filter area of 0.1 m^2) membrane at 0.5 $\text{m}^3 \cdot \text{h}^{-1}$. The highest ethanol productivity of 13.7 g/l/h was observed with a $D = 1.3 \text{ h}^{-1}$ with only 46% of the lactose in the feed being utilized.

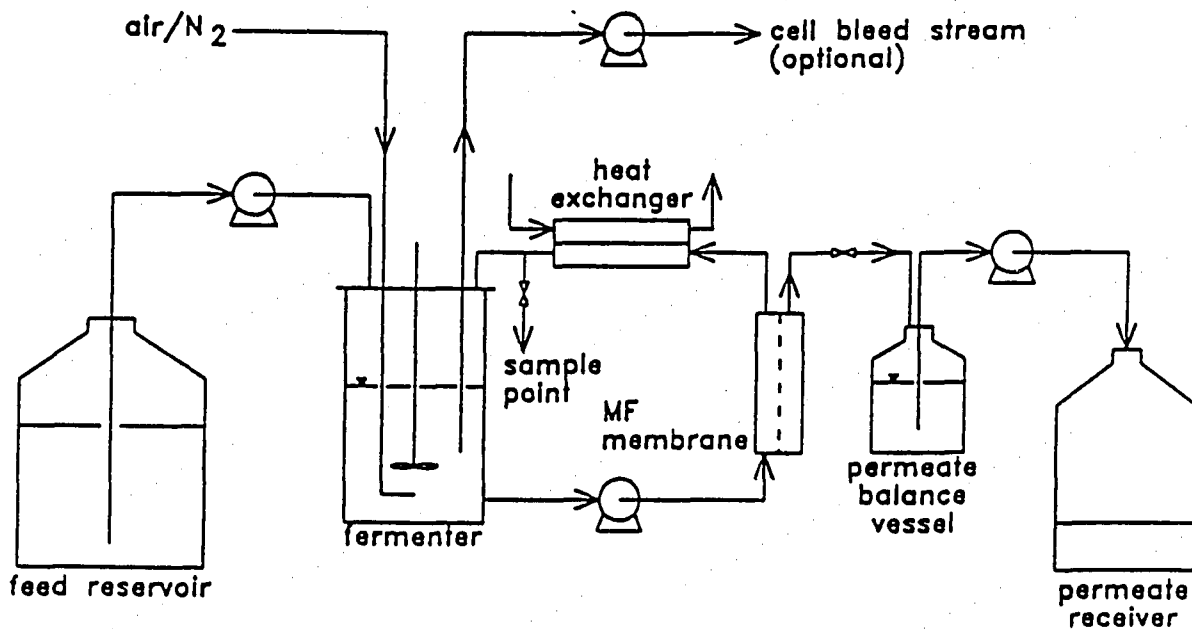


Figure 7. Schematic diagram of a membrane recycle fermentor (90).

Tower fermentor

The concept of continuous tower fermentation was developed for the brewing industry in Britain in the mid 1960's. Some yeasts naturally aggregate into flocs, which allow them to settle against an upflow of fluid. By incorporating a settling zone within the fermentor, free of turbulence caused by the evolving CO_2 , it is possible to retain yeasts in the fermentor simply and easily achieve high cell densities. With 120 g/l glucose in the feed, a 100% conversion was reached with a productivity of 13.9 g/l/h with a residence time of 4 hours in a tower fermentor constructed with 7.5 cm internal diameter and a height to diameter ratio of 22:1 (78) (Figure 8).

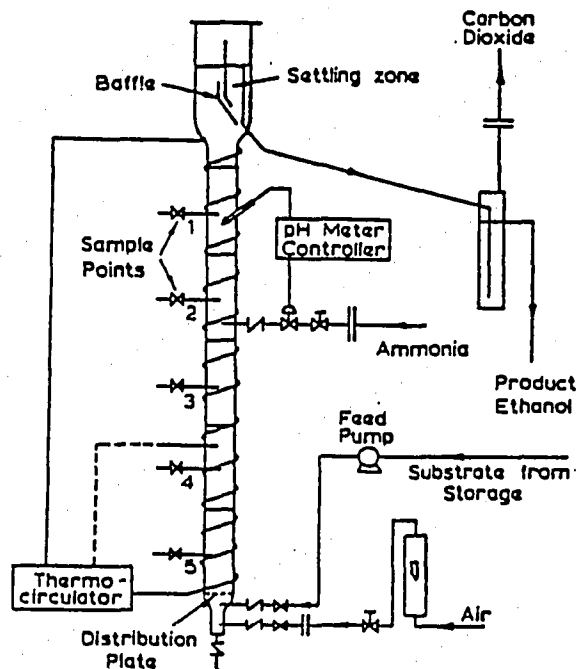


Figure 8. Schematic diagram of a tower fermentor (78).

Immobilized column reactor without support material

Schizosaccharomyces pombe, a naturally flocculating yeast, was cultivated in a column reactor to be used for continuous ethanol production by Hsiao and co-workers (40). An ethanol productivity of 87 g/l/h was reported with a feed of 150 g/l of glucose. Yeast flocs underwent morphological changes to heavy particles of 0.1-0.3 cm in diameter and the reactor was reported to be stable over a two-month period.

Continuous fermentation with cell recovery

Hollow fiber fermentor

A tubular bioreactor with continuous strands of hydrophobic microporous hollow fibers with extracting solvent flowing in a fiber lumen was used for yeast fermentation of glucose to ethanol. Yeast was immobilized on the shell side of the hollow fibers. The solvent extraction was carried out by two solvents (oleyl alcohol and dibutyl phthalate). The outlet glucose concentration decreased with an increase in solvent/substrate flow ratio. This effect of *insitu* extraction was stronger with oleyl alcohol than with dibutyl phthalate. (48).

Continuous dynamic immobilized biocatalyst bioreactor

Chen and Wayman (16) have used a continuous dynamic immobilized biocatalyst bioreactor (CDIBB) by co-immobilizing yeast and cellulases on glass fibre discs (Figure 9). The yeast was attached to glass fiber support by entrapment with sodium alginate and calcium chloride. The cellulase enzymes were precipitated on the surfaces of the

cells with tannin and glutaraldehyde. Cellulose, prepared from Aspen poplar (*Populus tremuloides*) by SO_2 -catalyzed prehydrolysis was used as a feed. Saccharification and fermentation occurred simultaneously in the bioreactor. The shelf-life of the system was about two weeks, with yields averaging 90% for the first 8 days and decreasing to 40% thereafter over a five day period.

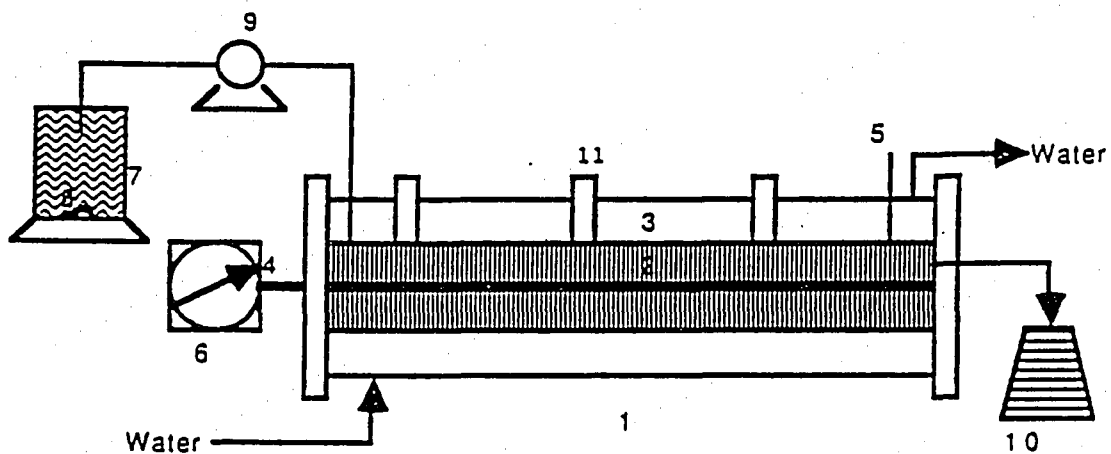


Figure 9. Schematic diagram of a continuous dynamic immobilized biocatalyst bioreactor (16).

- | | | |
|------------------------|----------------------|---------------------|
| 1. Bioreactor | 2. Glass fibre discs | 3. Heat exchanger |
| 4. Shaft | 5. Thermometer | 6. Motor |
| 7. Substrate reservoir | 8. Magnetic stirrer | 9. Peristaltic pump |
| 10. Product reservoir | | |

Fluidized bed fermentor

Continuous ethanol fermentation using a fluidized-bed reactor was studied by Nguyen and Shieh (73). The bioreactor consisted of a glass tube partially filled with spherical glass beads to distribute the flow and to retain the microcarriers containing the yeast. A peristaltic pump recycled the flow from the reactor into a container where the pH and other environmental conditions were monitored (Figure 10). Growth on the

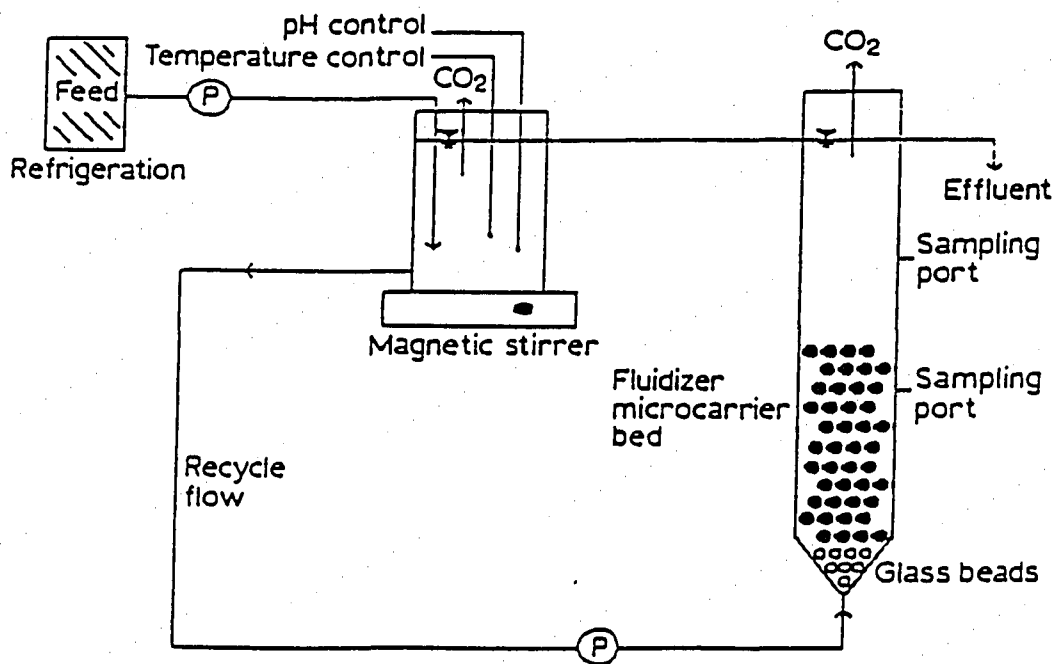


Figure 10. Schematic diagram of a fluidized bed fermentor (73).

celite R-633 microcarriers was established by adding a concentrated yeast culture to the reactor and operating it in a recycle mode for 14 days. After 2 weeks, continuous fermentations were started and performed at different dilution rates. The system reached a steady state in 72 days. The highest ethanol yield of 0.36% was obtained at a dilution rate of 0.15 h^{-1} . The average rate of ethanol production peaked between 0.11 and 0.14 h^{-1} . The system was operated for more than 226 days. It was not sterilized before the start of fermentation but it was disinfected with soap solution and hot water.

Multistage fluidized bed bioreactor

Tzeng and Fan (94) built a multistage fluidized bed by using *Saccharomyces carlbergensis* cells immobilized in sodium alginate beads (Figure 11). After gelation, the calcium alginate beads were treated in 1% triethylene tetramine for 1 h and in 1% glutaraldehyde for 4 min. These treatments improved the mechanical strength of the alginate beads and prevented the precipitation of calcium ions in the alginate matrix. The different stages of the fluidized bed reactor were separated by placing a 100 mesh sieve in the reactor. The volumetric productivities ranged from 14.90 to 17.41 g/l/h with 87 to 97% conversion, respectively. The outlet ethanol concentrations ranged from 66.8 to 93.3 g/l depending on the number of stages of the bioreactor.

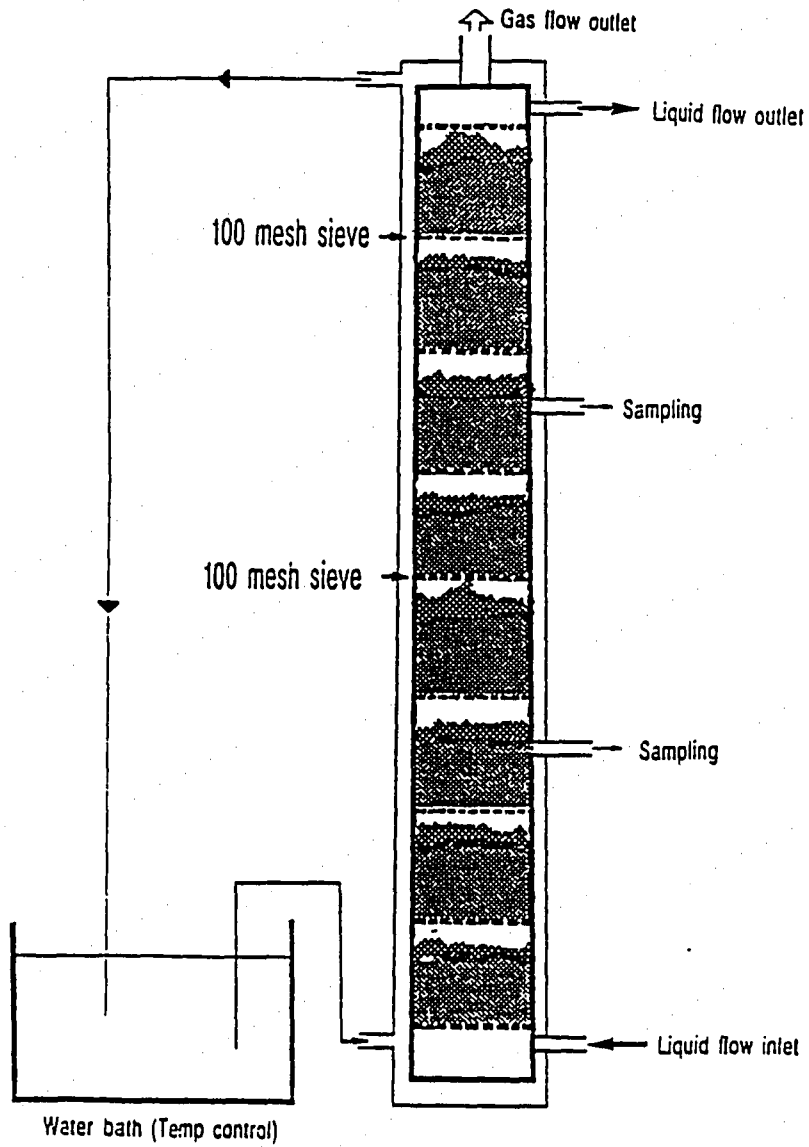


Figure 11. Schemtaic diagram of a multistage fluidized bed fermentor (94).

Solid substrate fermentation

Solid-phase fermentation

A semi-continuous solid-phase fermentation system was developed by Gibbons and coworkers (32) to produce fuel ethanol from sweet sorghum. In this process, dried and shredded sweet sorghum was rehydrated to 70% moisture, acidified to Ph 2.0 to 3.0, and inoculated with a spray of yeast culture. An optional pasteurization step (12 h at 70 to 80°C) was included prior to the inoculation step. The entire pulp, inoculated with the culture, was fed into an auger. Repeated batches of pulp inoculated with yeast were added to the fermentor and after 72 h of retention time the first batch of pulp added to the fermentor contained approximately 6% (v/v) ethanol. Due to the length of the auger and the slow rate of rotation, the pulp did not exit from the fermentor for 72 h, permitting complete fermentation of the available sugars. A similar model of solid-phase fermentation for production of ethanol and distiller's wet feed from fodder beets was designed by Gibbons *et al.* (32). The fermentation time of 24 h was sufficient to completely ferment the beat pulp into 8-9% ethanol (v/v). Based on the results of this study they proposed a model plant for continuous process for converting fodder beets into ethanol (Figure 12).

Extractive fermentation

Extractive fermentation is a process in which the fermentation and product recovery are integrated into a single step to overcome end product inhibition. An inert and biocompatible organic solvent such as oleyl alcohol and polypropylene glycol is

introduced into the fermentor to selectively extract ethanol. The ethanol is recovered from the solvent by means of flash evaporation, and the solvent is recycled to the fermentor. The distinguishing feature of the extractive fermentation process is the solvent regeneration and ethanol recovery. The ethanol-laden solvent leaving the fermentation vessel is sent to a flash vaporization unit, where, in a single equilibrium stage, a highly concentrated ethanol-in-water product (60-70% [w/v] ethanol) is obtained. The product stream is then sent to a distillation unit, while solvent having a considerably reduced ethanol content (> 85% removed) is returned to the fermentor after cooling (Figure 13). A feed concentration of 18% (w/v) fermentables and a dilution rate of 0.20 h^{-1} was found to provide the lowest ethanol production cost with an ethanol productivity of 16.3 g/l/h and a concentration of 82.0 g/l (27).

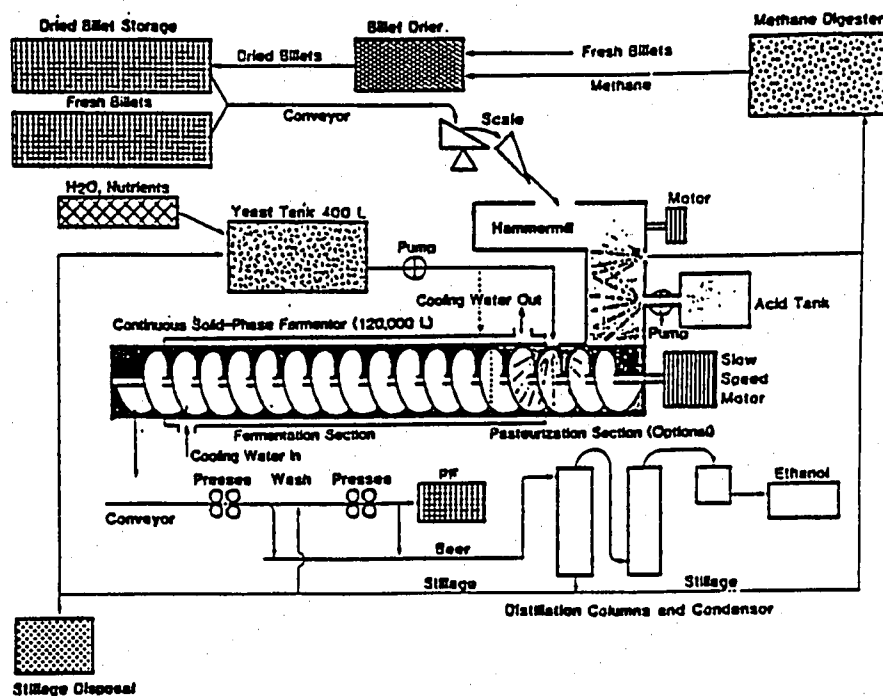


Figure 12. Schematic diagram of a plant for continuous solid-phase fermentation (32).

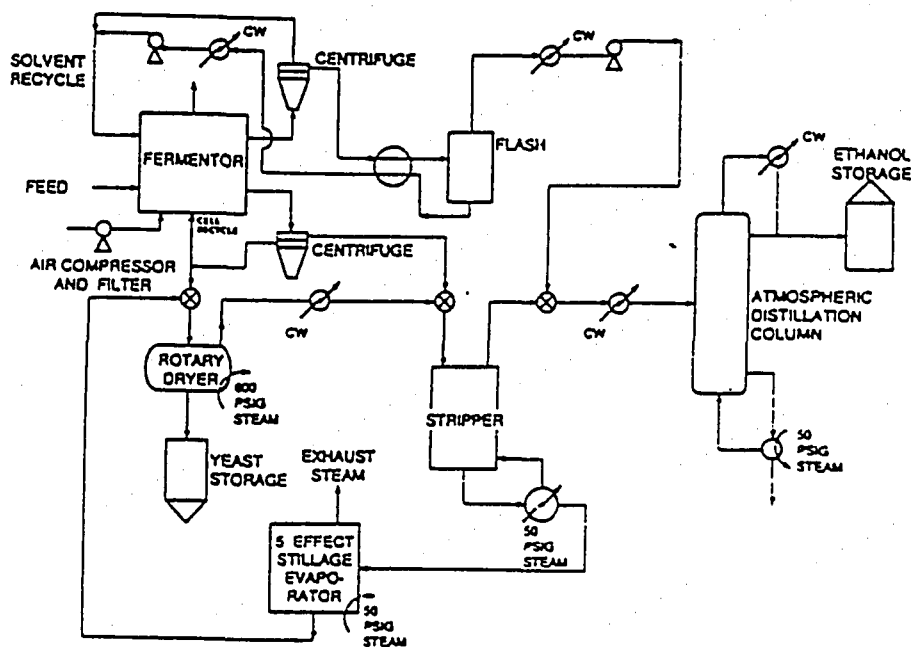


Figure 13. Schematic diagram for ethanol production using extractive fermentation (27).

Membrane extractive fermentation

Christen *et al.* (19) have developed a liquid membrane system for the extraction of ethanol during semicontinuous fermentation with *Saccharomyces bayanus*. The membrane consisted of a porous teflon sheet as support, soaked with isotridecanol for removal of ethanol (Figure 14). This system permitted combining biocompatibility, permeation efficiency and stability. The removal of ethanol from the fermentation broth

decreased inhibition, and resulted in the conversion of 452 g/l of glucose versus 293 g/l glucose without extraction. Membrane extraction resulted in 2.5 times increase in volumetric ethanol productivity. This process combined three operations: fermentation, extraction, and re-extraction (stripping the ethanol from solvent).

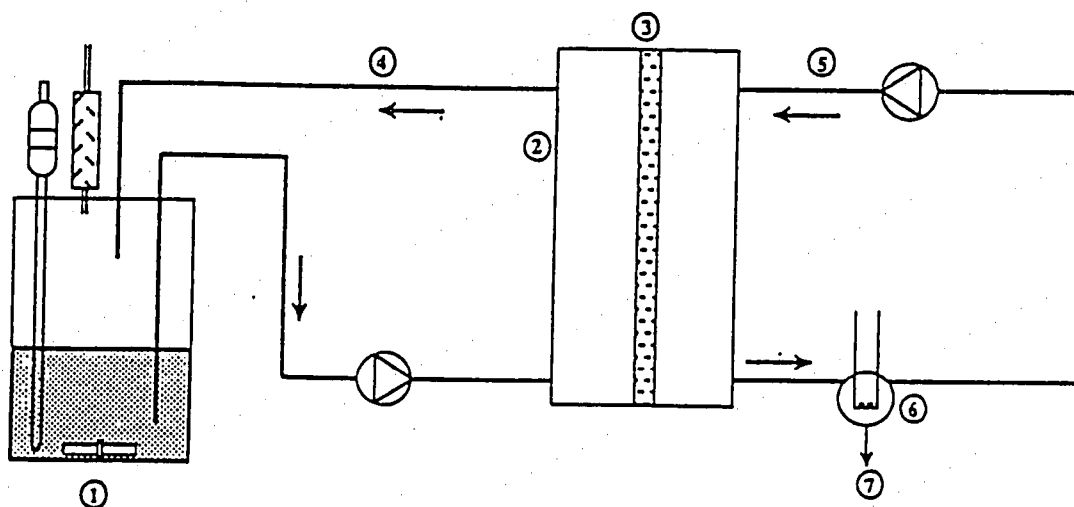


Figure 14. Schematic diagram of an extractive fermentor (19).

- | | | |
|-----------------------|----------------------------|------------------------------|
| 1. Fermentor | 2. Permeation cell | 3. Supported liquid membrane |
| 4. Extracted phase | 5. Gaseous stripping phase | 6. Cold trap |
| 7. Condensed permeate | | |

Vacuum fermentation

The continuous removal of ethanol from the fermentation broth eliminates ethanol inhibition. This has been achieved by operating the fermentor under vacuum (Figure 15). Ethanol productivities of 82 g/l/h were obtained by Ramalingham *et al.* (79) using this vacuum fermentation. An added advantage of vacuum fermentation is

the production of a more concentrated ethanol stream, which reduces some distillation requirements, to produce 190 proof ethanol. A major constraint of vacuum fermentation, however, is the accumulation of the toxic, non-volatile components within the fermentor. To avoid yeast inhibition by non-volatile components, a medium bleed of the fermentor must be continuously withdrawn. The cell recycle and vacuum operation may increase the likelihood of microbial contamination. Furthermore, additional capital and operating costs for both systems and limited reliability makes these processes unsuitable for industrial scale operations.

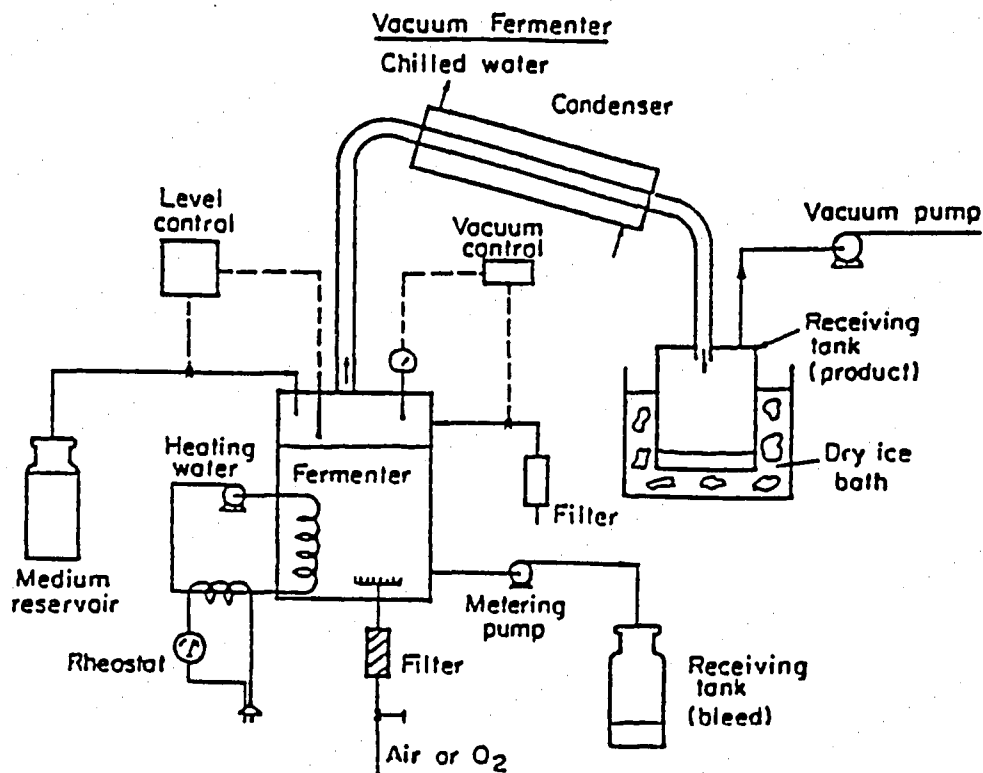


Figure 15. Schematic diagram of a vacuum fermentor (79).

Integrated fermentation unit

Immobilized yeast reactor coupled with membrane pervaporation unit

Pervaporation is an evaporation process utilizing a semi-permeable membrane through which the medium containing the solvent to be evaporated flows. The entire system, under vacuum, increases the recovery of the product. A system comprising of an immobilized yeast reactor producing ethanol, with a membrane pervaporation module for continuously removing and concentrating the produced ethanol, was developed by Shabtai and coworkers (Figure 16) (83). The yeasts were immobilized in a cross-linked polyacrylamide-hydrazide gel coated with calcium alginate and extruded as 5 mm beads. An ethanol productivity of 20 to 30 g/l/h was achieved in continuous operation (over 40 days). Membrane fouling was prevented by short washing steps and by using two different modules that were interchanged in and out of the bioreactor.

Biofilm bioreactors for ethanol production

A biofilm reactor is a system in which growth takes place on an inert surface producing a film of microorganisms which is in direct contact with the surrounding medium and is immobilized (11). Not all microorganisms are film formers but nonfilm forming organisms are naturally entrapped or immobilized in the biofilms. In other words, biofilms are a natural mechanism for cell immobilization. Biofilm reactors present several advantages over entrapped systems. A culture is inoculated into the fermentor which contains specific inert supports and the cells are allowed to grow forming a biofilm on the support surfaces. This obviates the need to use special

techniques to entrap cells into the matrices prior to use in the reactor. The biofilm is then maintained by the film former, requiring no special maintenance schedule for long term use. The combination of high conversion, high product concentrations and high productivity is essential to the economic production by fermentation. Use of biofilm reactors offers a combination of these factors.

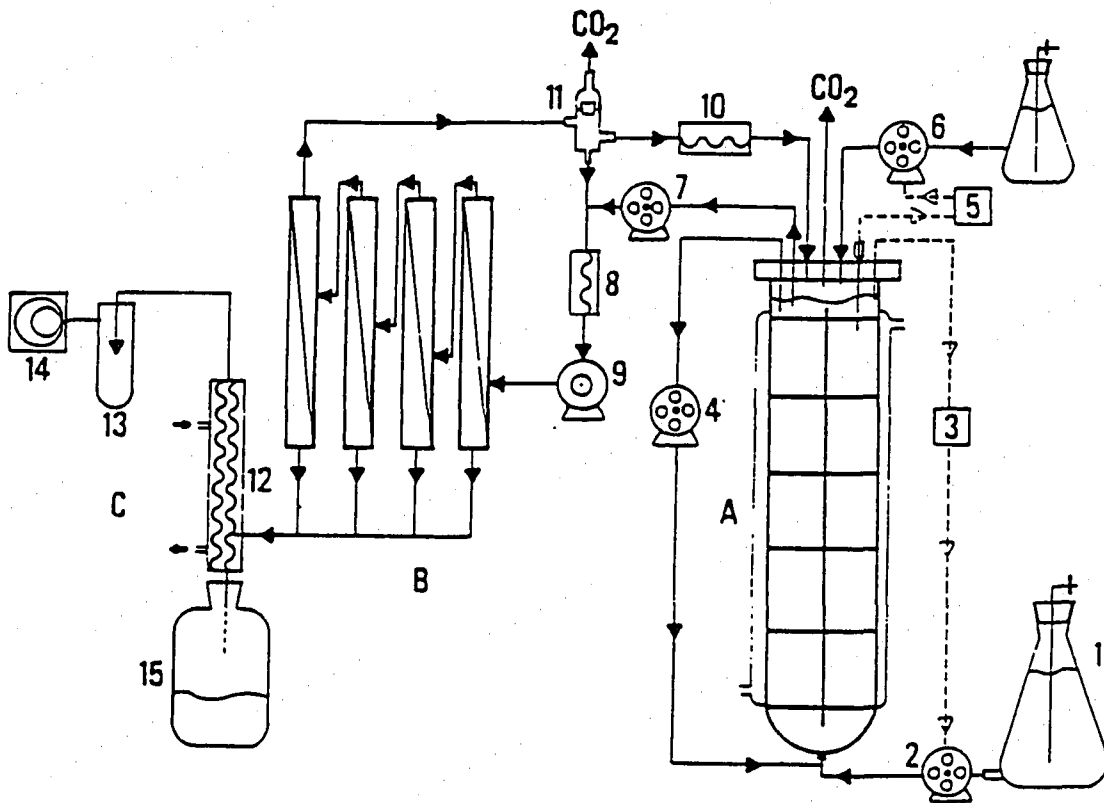


Figure 16. Schematic diagram of an immobilized yeast reactor coupled with pervaporation unit (83).

- | | | |
|----------------------------|--------------------------------|--------------------------|
| A. Immobilized reactor | B. Membrane pervaporation unit | c. Product recovery unit |
| 1. Feed reservoir | 3. Level controller | 5. Liquid hold tank |
| 11. Pressure relief device | 13. Liquid nitrogen trap | 14. Vacuum pump |
| 8,10,12. Heat exchangers | 2,4,6,7,9. Pumps | 15. Product reservoir |

Biofilm reactors result in high cell densities, high productivities, reduced diffusional resistance, less inhibition and long operating time with little maintenance.

Packed bed fermentor

Saccharomyces cerevisiae cells immobilized in 3% κ -carrageenan were used to build a packed-bed reactor for use in continuous ethanol fermentation. A tapered-column reactor was used to offset the problem of pressure build-up and channelling caused by evolving CO₂ and aeration was used. With a cell loading of more than 40 g/l, an ethanol productivity of 21.1 g ethanol/l gel/h was obtained. Feeding oxygen into the reactor improved the yield by supplying a cellular nutrient, by decreasing the interface mass-transfer resistance caused by micromixing, by redispersing the settled biomass, and by breaking the clusters of beads held together with the accumulating cells (37).

Plug flow bioreactor

Das and coworkers (26) have used lignocellulosic materials for whole cell immobilization in developing a vertical packed column reactor (Figure 17). Four different lignocellulosic materials (bagasse, sawdust, rice-husk and rice-straw) were used for immobilization of yeast cells. Immobilization was achieved by recirculating a concentrated cell suspension for 12 h. A maximum productivity of 17.4 g/l/h corresponding to a dilution rate of 0.39 h⁻¹ and ethanol concentration of 45.8 g/l was reported with rice-straw as the solid matrix for immobilization. A decline in ethanol

productivity was reported after 22 days of continuous operation. Different types of reactor configurations were studied to overcome the disruption effect caused by the generation of the large volume of CO_2 in the cylindrical column bioreactor. Increased productivities were obtained when reactors with rhomboidal and tapered column shapes were used.

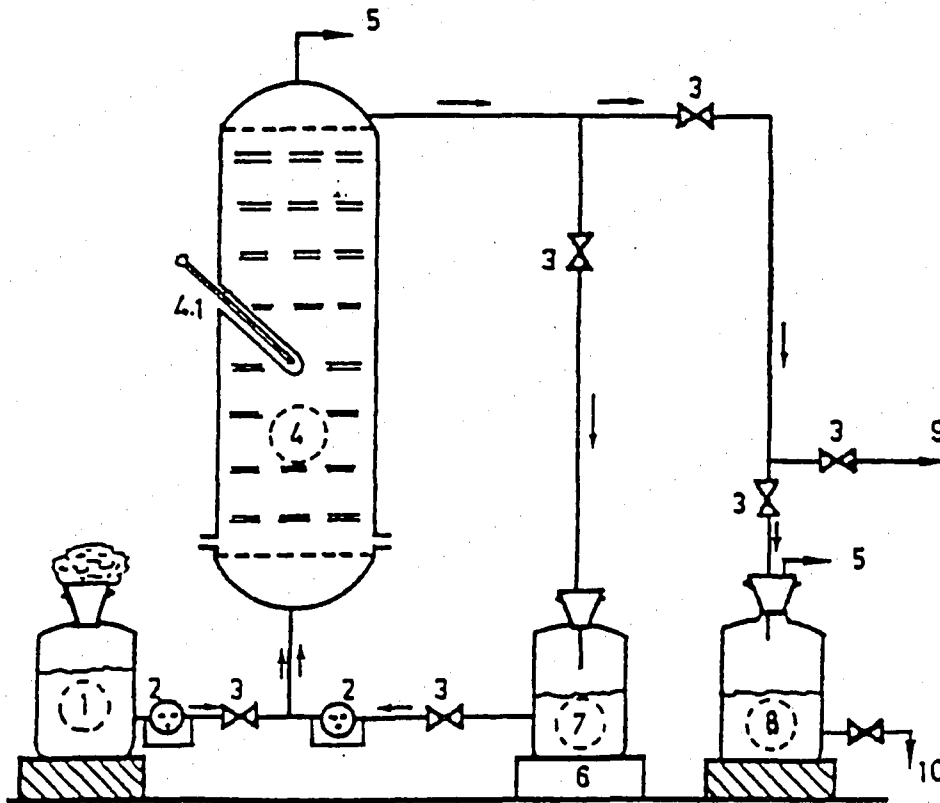


Figure 17. Schematic diagram of a vertical packed bed reactor (26)

- | | | |
|-----------------------------|-------------------------|-----------------------|
| 1. Medium reservoir | 2. Peristaltic pumps | 3. Valves |
| 4. Immobilized cell reactor | 4.1 Thermometer | 5. CO_2 vent |
| 6. Magnetic stirrer | 7. Yeast cell reservoir | 8. Product reservoir |
| 9. Sampling point | 10. Drain | |

Packed-column reactor

A packed-column reactor with yeast cells immobilized in calcium alginate gel was used to study the fermentation variables involved in continuous ethanol fermentation. A maximum ethanol concentration of 98 g/l with a mean residence time of 3.8 h was reported. Under steady-state conditions ethanol productivity was 38 g/l/h. The performance of the packed bed reactor was considerably better than that of a CSTR. After 20 days of continuous fermentation, some deactivation of cells occurred, but ethanol productivity was recovered by reactivating the cells with nutrients. It was reported that initial activation as well as intermittent reactivations during the fermentation were very important to the satisfactory performance of the reactor system over a prolonged period (56).

Rotating biological surface bioreactor

A rotating biological surface (RBS) reactor, described as a trickling filter, was used for the continuous production of ethanol by Del Borghi and coworkers (Figure 18) (30). A synthetic commercial sponge was used to trap the yeast cells on the disks. Ethanol productivity of 7.1 g/l/h at a dilution rate of 0.3 h^{-1} was reported in the RBS. This was 2.5 times greater than the maximum productivity obtained at a lower dilution rate in the RBS reactor without support.

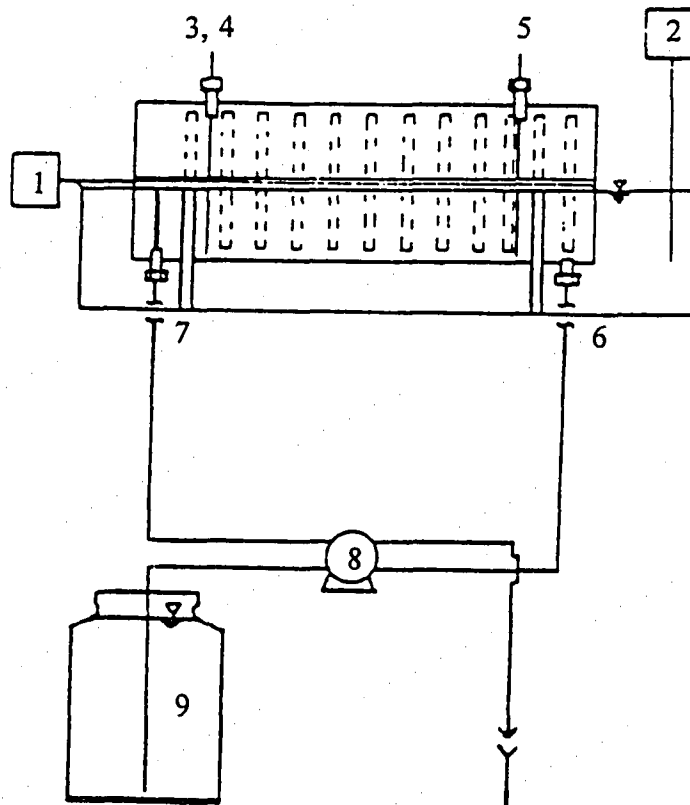


Figure 18. Schematic diagram of a rotating biological surface bioreactor (30).

- | | | |
|------------------|--------------------------|---------------------------------|
| 1. Shaft stirrer | 2. Temperature regulator | 3, 4. pH control and regulation |
| 5. Thermometer | 6. Input | 7. Output |
| 8. Pump | 9. Nutrient reservoir | |

Rotating fiber disc fermentor

Continuous ethanol production from glucose, sucrose, cane molasses and corn wet-milling waste by *Saccharomyces cerevisiae* and *Pichia stipitis* was reported by Parekh and Wayman (75). The cells were immobilized on rotating fiber discs by calcium alginate in a continuous dynamic immobilized cell bioreactor (CDIR). Glucose

and sucrose at 56 g/l was fermented in 1.6 h with about 90% substrate utilization. Waste water from a corn wet-milling process with 115 g/l sugars was completely fermented in 2.5 h by *S. cerevisiae* loaded at 56 g/l cell density. Cane molasses containing 195 g/l total sugars was fermented in 3.6 h with an ethanol productivity of 23 g/l/h and a 90% substrate utilization. The estimated half life of the bioreactor was 98 days (figure 19).

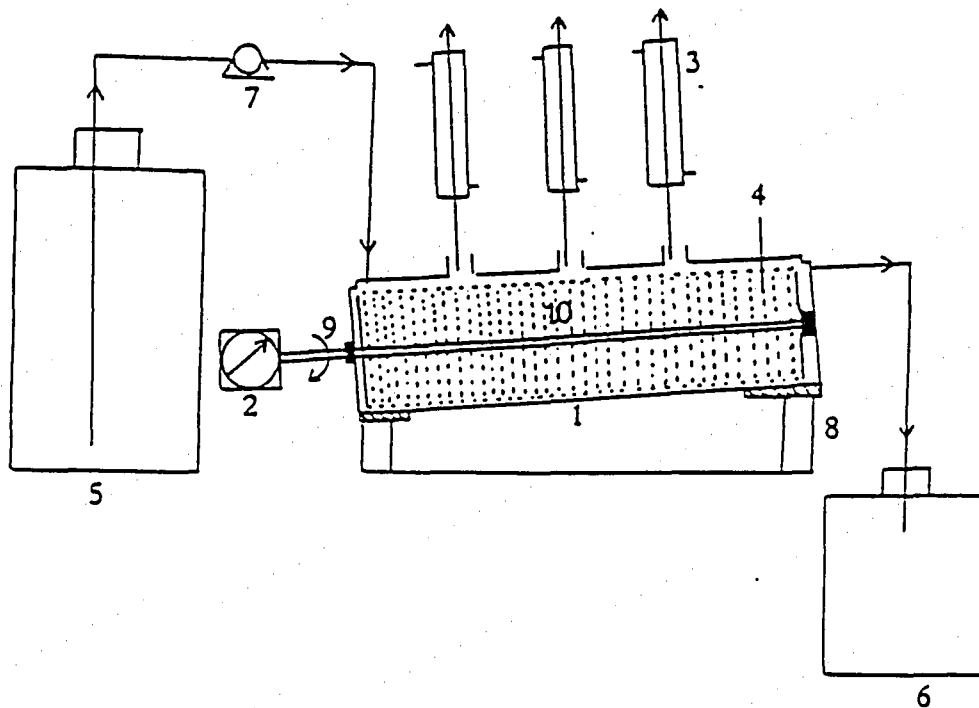


Figure 19. Schematic diagram of a rotating disc fermenter (75).

- | | | |
|-----------------------|-------------------|----------------------|
| 1. Rotating fermentor | 2. Motor | 3. Condenser |
| 4. Thermometer | 5. Feed reservoir | 6. Product reservoir |
| 7. Pump | 8. Stand | 9. Central shaft |
| 10. Fiber discs | | |

Rotary drum fermentor

Ethanol production from sweet sorghum by solid-state fermentation (SSF) in a rotary-drum fermentor was reported by Kargi (49). This method involves chopping the sorghum pith into fine particles and then adding a concentrated yeast inoculum.

Fermentation occurs on the surface of the sorghum particles, which can be regarded as a single, solid phase. A rotary drum offers an inexpensive means for mixing the fermenting sorghum, thereby ensuring homogeneity and effective heat transfer. To ensure anaerobic conditions in the fermentor the yeast inoculum was mixed with sodium sulfide. The rate of ethanol formation decreased with increasing rotational speed of the drum. The maximum rate and extent of ethanol formation was 3.1 g/l/h at 1 rpm rotational speed with a disc diameter of 22 cm and length of 15 cm. Kargi and Curme (49) have developed a rotary-drum fermentor for the solid-state fermentation of sweet sorghum to ethanol. They reported a maximum ethanol productivity of 3.1 g/l/h. Gibbons and co-workers (31) used a semi-continuous solid-phase fermentation for production of fuel ethanol from sweet sorghum. In this process dried and shredded sweet sorghum rehydrated to 70% moisture was fermented in 72 h, with ethanol yields of 176 to 179 liters per 1000 kg of dry sweet sorghum.

EVALUATION OF PLASTIC-COMPOSITE SUPPORTS FOR ENHANCED ETHANOL PRODUCTION IN BIOFILM REACTORS

A paper to be submitted to the Journal of Industrial Microbiology

Mahipal Reddy Kunduru¹ and Anthony L. Pometto III^{1,2}

Abstract

Biofilms are a natural form of cell immobilization that result from microbial attachment to solid supports. Biofilm reactors with polypropylene composites containing up to 25% (w/w) of various agricultural materials (corn hulls, cellulose, oat hulls, soybean hulls or starch) and micronutrients (soy bean flour or zein) were used for ethanol production. Plastic composite supports were prepared by a high temperature extrusion of polypropylene and agricultural material into 2-3 mm chips. Pure-cultures of *Zymomonas mobilis* (ATCC 31821) or *Saccharomyces cerevisiae* (ATCC 24859) and mixed-cultures with either of the ethanol producing microorganism and the biofilm forming *Streptomyces viridosporus* T7A (ATCC 39115) were evaluated. Pure- and mixed-culture combinations were evaluated in continuous fermentation in glucose-yeast extract medium in a bioreactor with a 20 ml working volume, 50 ml plastic composite supports and dilution rates of 0.18 to 10.5 h⁻¹.

An ethanol productivity of 374 g/l/h with a 44% yield was obtained on

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soybean hull-zein-polypropylene composite supports using *Z. mobilis* in pure-culture with 10% glucose feed. The ethanol productivity obtained is the highest reported to date. With mixed-culture fermentations employing *Z. mobilis* and *S. viridosporus*, an ethanol productivity of 147.5 g/l/h was obtained on corn starch-soybean flour composite support. With pure-culture fermentation with *S. cerevisiae*, maximum productivity of 40 g/l/h with a 47% yield was obtained on soybean hull-soybean flour plastic composite support. Mixed-culture fermentation using *S. cerevisiae* and *S. viridosporus* resulted in ethanol productivity of 190 g/l/h with a 35% yield, when oat hull-polypropylene composite supports were used. The maximum productivities obtained in continuously stirred reactors without supports were 124 g/l/h and 5.4 g/l/h with *Z. mobilis* and *S. cerevisiae*, respectively. These productivities are significantly lower than the productivities obtained with biofilm reactors using composite supports. Percent yields were generally lower with mixed-culture fermentations than those observed with pure-culture fermentations. Biofilm formation on the chips was detected by the change in weight and Gram staining of the support material at the end of the fermentation. Ethanol production rate and concentrations were consistently higher in biofilm reactors than in suspension cultures. Biofilm reactors with pure culture of *Z. mobilis* had higher ethanol productivity, whereas, biofilm reactors with mixed-cultures of *S. cerevisiae* and *S. viridosporus* resulted in higher productivities. Fermentations with *S. cerevisiae* resulted in lower productivities than fermentations with *Z. mobilis*.

Introduction

Ethanol is a primary alcohol that can be produced by chemical synthesis from petrochemical feedstocks and by microbial fermentation using renewable plant products. Ethanol is used as a motor fuel additive, most commonly in a blend with gasoline known as gasohol. The chemical industry uses ethanol as a feedstock and as a solvent. Ethanol can also be fermented to acetic acid, which is used as a food acidulant and as a road deicer as CMA (calcium-magnesium-acetate) (1). Ethanol is considered appropriate as a turbine fuel for peak load electric utilities requirements (30). In the U.S, more than half of the denatured alcohol is sold as solvent for nitrocellulose coatings, shellacs, inks, hydraulic fluids, liquid detergents, soaps, deodorants, perfumes, antiseptics and lotion. Undenatured ethanol is used by the cosmetic, pharmaceutical and food industries in the production of vitamins, flavors and essences, mouthwashes, blood products and fortified wines (14) and as growth substance for single cell production (9).

Ethanol production costs by fermentation were less than \$1.25 per gallon in 1992, depending on the process used and feedstock costs (14). Raw materials are the major costs representing up to 70% of the final price (24). Conventionally, ethanol has been produced by batch fermentation, which has the drawbacks of large fermentor volume requirements, low productivity, difficulty in automation and high operating costs. To improve fermentation several techniques have been applied. These include vacuum fermentors (10), cell recycling (27), hollow fiber membrane reactors with recycling (7) and immobilization of cells (5, 8). Various immobilization procedures such as covalent coupling (including treatment with cross-linking agents), adsorption on

to solid inert carriers, and entrapment in semi-permeable inert supports such as hydrogels, fibers and membranes were used. Supports such as κ -carrageenan gels (15), calcium alginate (18), ion exchange resins (20), vermiculite (4) and γ -alumina (19) were used for cell immobilization. Viable cells immobilized in solid gel matrices (i.e. calcium alginate) as beads have been studied in packed bed (32) and fluidized bed reactors (25). However, these systems have relatively low efficiency, and find limited applications due to the diffusional resistance of substrate or product and rapid removal of CO₂ from the reactor, in addition to decreased microbial viability for long term production of ethanol. Improving industrial fermentation productivity requires development of increased production rates with reduced fermentor volumes and decreased operating costs.

Biofilms are a natural form of cell immobilization that results from microbial attachment to solid supports (6). Biofilms have been used in waste water treatment plants (21), for production of vinegar by the "quick vinegar process", mineral ore treatment (9) and lactic acid production (12). This paper describes the use of biofilm reactors which use plastic composite supports for enhanced ethanol production. A three-fold increase in ethanol productivity was obtained in biofilm reactors containing plastic composite supports with *Z. mobilis* as ethanol producer. Fermentations were performed with *Z. mobilis* or *S. cerevisiae* as the ethanol producers in pure-culture and with either of the ethanol producer and *S. viridosporus* as the biofilm former in mixed-culture fermentations.

Materials and Methods

Microorganisms and media. *Zymomonas mobilis* (ATCC 31821) was maintained in a medium containing 2% (w/v) glucose, 0.5% (w/v) yeast extract (Difco Laboratories, Detroit, MI), 0.2% (w/v) $(\text{NH}_4)_2\text{SO}_4$, 0.05% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2% (w/v) KH_2PO_4 at 4°C and was subcultured every two weeks. *Saccharomyces cerevisiae* (ATCC 24859) was maintained on a medium containing 2% (w/v) glucose, 1.0% (w/v) yeast extract (Difco Laboratories) and 2% (w/v) peptone (Difco Laboratories) at 4°C and subcultured every 4-6 weeks. Biofilm former *Streptomyces viridosporus* T7A (ATCC 39115) was maintained on 0.6% yeast extract agar slants at 4°C for 3-6 weeks (22).

For continuous fermentation the medium for *Z. mobilis* had 0.5% (w/v) yeast extract (Difco), 0.2% (w/v) $(\text{NH}_4)_2\text{SO}_4$, 0.05% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.2% (w/v) KH_2PO_4 . A glucose concentration of 10 and 12% (w/v) was used for pure- and mixed-culture fermentation, respectively. Medium used for yeast fermentations contained 0.6% (w/v) yeast extract (Difco), 0.023% (w/v) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15% (w/v) KH_2PO_4 and 0.4% (w/v) $(\text{NH}_4)_2\text{SO}_4$. The glucose concentration was 7.5 and 10% with pure- and mixed-culture fermentations, respectively. The phosphate salts were autoclaved separately and then added to the medium aseptically before fermentation was started.

Support materials. Various plastic composite supports containing agricultural materials (25% w/w) were used as solid supports (Table 1). The plastic composite supports were prepared by high-temperature extrusion of the polypropylene (Quantum USI Division, Columbus, OH) and agricultural materials in a Brabender PL2000 counter-rotating twin-screw extruder (C. W. Brabender Instruments, Inc., South Hackensack, N.J) by using the method of Demirci *et al* (12). The barrel temperatures were 200, 210 and 220°C, the die temperature was 220°C and the screw speed was 20 rpm. The agricultural products used were cellulose (Sigma Chemical Co., St. Louis, MO), corn starch (American Maize-Products Co., Cedar Rapids, IA), ground (20 mesh) oat hulls (National Oats Co., Cedar Rapids, IA), soybean flour (Archer Daniel Midland Co., Decatur, IL), ground (20 mesh) corn hulls (Penford Products Co. Cedar Rapids, IA), and zein (Sigma Chemical). Each agricultural material was vacuum dried for 48 h at 110°C prior to being used for extrusion. Polypropylene pellets and specific agricultural blends were mixed for several minutes in a container and then added to the extruder hopper. Polypropylene was compounded with different levels and blends of agricultural materials. The melted polypropylene was uniformly mixed with agricultural product by the counter-rotating movement of the twin-screws and extruded as 3 mm diameter rods, air cooled and then cut into chips of 2-3 mm in length with a pelletizer. Polypropylene extruded with protein containing agricultural material was difficult to extrude and was charred by the high temperatures employed.

Evaluation of biofilm. The biofilm formed on the support material was evaluated by weight change, by clumping after drying at 70°C overnight, and by gram staining the chips. After drying the supports in a flask, were shaken vigorously to evaluate chip-clumping strength (13). Supports with good biofilm resisted separation, whereas supports without any biofilm formation separated easily. Gram staining was performed on supports after the fermentation and the resulting color development was compared visually with the color of uninoculated Gram stained supports.

Continuous ethanol fermentation. Fermentation was carried out in 60 ml plastic syringes with an estimated working volume of 20 ml by using the method of Demirci *et al.*(12) (Figure 1). A 9-liter carboy containing 4.5 to 6 liters of sterile medium was fed into the syringe at its needle port. A T-connector in the feed line was used to supply filter sterilized air for yeast and streptomycetes or nitrogen for bacterium. The wide mouth of the syringe was fitted with a silicone stopper that contained two glass tubes. One port was covered with a septum and used for inoculation, and the other was used as an exit line. The system contained liquid breaks in the feed and exit lines to prevent contamination of the medium reservoir and the reactors during sampling or during changing the medium for the mixed-culture fermentations. The syringe was filled with 50 ml (average weight of 18.65 g and density of 0.373 g/cc) of a plastic composite support and was clamped tightly with the silicone stopper at the wide mouth end, then sterilized by autoclaving at 121°C for 30 minutes. Specific culture medium was sterilized by autoclaving at 121°C for 85 minutes and then aseptically connected to

each reactor. For mixed-culture fermentation the reactors were inoculated with 1 ml of *S. viridosporus* spore suspension ($\sim 1.0 \times 10^9$ spores/ml). Each reactor was incubated in batch fermentation at 37°C for 24 h and then changed to continuous fermentation at a dilution rate of 0.18 h⁻¹ for 10 days to develop a biofilm. The medium was switched and the reactors inoculated with 1 ml of the ethanol producing bacterium or yeast and incubated at 30°C. A 24 h batch fermentation was followed by a continuous fermentation at various dilution rates (0.08, 0.36, 0.72, 1.44, 2.88, 5.76, 0.48, 0.96, 1.92, 3.84, 7.68, 0.66, 1.32, 2.64, 5.28, 10.56 h⁻¹). The fermentation was anaerobic when *Z. mobilis* was the ethanol producer. Each dilution rate was maintained for 24 hours and samples were collected at 5-6 hour intervals. The control reactor contained polypropylene supports in pure- and mixed-culture fermentations and without supports in pure-culture fermentations. Reactors without supports with a working volume of 20 ml contained a magnetic stir bar to prevent culture from settling at the bottom for the *Z. mobilis* fermentation. Each continuously stirred reactor (CSR) was suspended in a 30°C water bath placed on a magnetic stir plate.

Analysis of culture broth. The suspended cell density in the reactors was measured by absorbance at 620 nm using a Bausch and Lomb Spectronic 20 spectrophotometer (Milton Roy, Rochester, NY). Percentage glucose and ethanol were measured by using a Waters High Pressure Liquid Chromatograph (Millipore Corporation, Milford, MA) equipped with a Waters Model 401 refractive index detector, column heater, autosampler and computer controller. The separation of ethanol, glucose

and other broth ingredients were done on a Bio-Rad Aminex HPX-8711 column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) using 0.012 N Sulfuric acid as a mobile phase at a flow rate of 0.8 ml/min with a 20 μ l injection volume and a column temperature of 65°C.

Results and Discussion

Percent Yield. The percent yield is a measure of the conversion efficiency of glucose to ethanol and is defined as ethanol produced divided by glucose consumed times 100. Theoretical yield for ethanol production is 51% (31). The percent yield for pure cultures of *Z. mobilis* ranged from 36 to 52% (Figure 2). Generally, the percent yields were lower for mixed-culture fermentations (Figure 3) than compared to pure-culture fermentations at the same dilution rates, suggesting that the biofilm former *S. viridosporus* utilized some of the glucose for cell maintenance and growth. There was no appreciable difference in the percent yields among the various composite supports tested. The yields were consistently higher with the plastic composite supports than the yields obtained from the controls with polypropylene alone or with suspension-culture fermentations at all the dilution rates tested in pure-cultures of *Z. mobilis*. With the bacterial mixed-culture reactors there was no appreciable difference in the yields among the polypropylene-alone and composite supports. Biofilm formation by the *S. viridosporus* on the polypropylene supports most likely retained the ethanolic

microorganism in the bioreactor. A similar pattern was observed for pure- and mixed-culture fermentations with *S. cerevisiae* (Figure 4 and 5). The percent yield for fermentation with pure- and mixed-culture of *S. cerevisiae* was much lower than that obtained with *Z. mobilis* with ranges from 8 to 47% for pure-culture fermentation and 15 to 38% with mixed-culture fermentation.

Productivity. The productivity (g/l/h) is a measure of ethanol production per hour (calculated as ethanol produced in g/l times the dilution rate in h^{-1}). Ethanol productivity was very low in suspension culture fermentations for both *Z. mobilis* and *S. cerevisiae* (Figure 2 and 4). For *S. cerevisiae*, the productivity improved in mixed-culture fermentations with pure polypropylene supports. Productivities were generally 4 to 9 times higher in pure-culture fermentations of *Z. mobilis* than compared to pure-culture fermentations of *S. cerevisiae*. A cell wash-out was not observed in any of the plastic composite support reactors with *Z. mobilis* fermentation, even at the highest dilution rate of $10.56 h^{-1}$ (Figure 2 and 3). The productivities were much lower in mixed-culture fermentations of *Z. mobilis*, which could be due to the continuous supply of air needed for the aerobic *S. viridosporus* to grow. The cell growth rate and ethanol productivity for *Z. mobilis* has been reported to decrease with increasing oxygen supply, with ethanol productivity being more sensitive to oxygen supply than the growth rate (28).

With *S. cerevisiae*, the productivities were higher in mixed-culture fermentations, with the plastic composite supports resulting in higher productivities than the suspension cultures or pure-polypropylene support reactors in both pure- and mixed-culture fermentations. The highest productivity of 364 g/l/h was obtained in pure-culture fermentation of *Z. mobilis* with soybean hull-zein-polypropylene composite supports (Figure 2) and a productivity of 149.5 g/l/h was obtained on corn starch-soybean flour-polypropylene composite material with mixed-culture fermentations (Figure 3). The highest productivity obtained with pure-cultures of *S. cerevisiae* was 40 g/l/h on soybean hull-soybean flour-polypropylene composite supports (Figure 4). The highest productivity obtained with mixed-culture fermentation was 190 g/l/h on oat hull-polypropylene and 150 g/l/h on oat hull-soybean flour-polypropylene (Figure 5). These productivities are significantly higher than those currently reported in the literature (Table 2).

Ethanol production. Ethanol and glucose concentrations for each dilution rate were analyzed from samples collected every 5-6 h over a 24 h period to determine the steady-state condition. Typically, a steady-state condition was observed after the first 10 h of continuous fermentation at each dilution rate tested. The ethanol concentrations were consistently higher for plastic composite support reactors in both pure- and mixed-culture fermentations than for cell-suspension cultures or reactors containing pure-polypropylene as support material. The cell densities from the effluents of the composite supports with *Z. mobilis* showed an absorbance (620 nm) of 1.17, 0.18 and

0.10 from soybean hull-zein, polypropylene alone and suspension culture reactors, respectively. This high cell density in the continuous fermentation effluent indicates enhanced cell growth. The agricultural material blends in the composite support provides some micronutrient to the microorganism and/or it provides a surface for cell attachment promoting biofilm development. Cell attachment was confirmed by the intense color of the Gram stained harvested supports. There was also a 10-15% increase in the plastic composite support's weight at harvest. Support materials from bioreactors illustrating the highest productivity also demonstrated excellent clumping, weight gain and retention of color on Gram staining. Further long-term studies are needed to evaluate the performance of these composite support materials for use in continuous fermentations.

Support Materials. In pure-culture fermentations with *Z. mobilis* the reactors containing soybean hull-zein, corn starch-soybean flour and cellulose-soybean flour plastic composite supports demonstrated high concentrations of ethanol and good biomass retention. The micronutrients (amino acids) in the soybean flour and zein of the composite supports have provided a better environment for the growth of the biofilm. In mixed-culture fermentations the reactors containing oat hull-zein and soybean hull-soybean flour plastic composite supports resulted in better ethanol productivities. In pure-culture fermentation with *S. cerevisiae* soybean hull-soybean flour, corn hull-zein and soybean hull-zein plastic composites performed better. Whereas, oat hull-zein and oat hull-soybean flour plastic composites had a better

performance in mixed-culture fermentation. In our preliminary studies with the various plastic composite supports and culture combinations, good yeilds and ethanol concnetrations were not obtained for every material. Subsequently only the supports that performed better than or equal to the polypropylene-alone composite support were further investigated.

These data suggests that the micoronutrients in the composite supports have improved ethanol fermentation by forming a better biofilm and thereby improving the rate of ethanol production. The presence of soybean flour, soybean hull and zein resulted in good biofilm formation and better productivities with *Z. mobilis* in pure culture fermentation. The addition of cellulosic agricultural material alone to the plastic composite support did not achieve the same results. Similarly with *S. cerevisiae* fermentations the reactors containing soybean hull, soy flour and zein formed better biofilms and resulted in increased ethanol production. *Z. mobilis* is a preferred organism for use in biofilm culture reactors because of it's high productivity and it's cell aggregation characteristics. Mixed-culture fermentations with *Z. mobilis* did not improve ethanol productivity, but did reduce yields. Therefore, mixed-culture fermentations with *Z. mobilis* and *S. viridosporus* is not recommended for use in biofilm reactors for ethanol production. However, mixed-culture fermentations may be considered with *S. cerevisiae* to obtain higher productivities but with greatly decreased yields. The type of plastic composite support material used in a bioreactor depends on the microorganism(s) used as demonstrated by the performance of different cultures employed in this research. Overall, these results indicate a tremendous gain in ethanol

productivities with biofilm bioreactors.

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Table 1. Composition of polypropylene composite supports^a.

PP-Composite chip	Major Agricultural product (%)	Minor Ag. product (5%)
Polypropylene	-	-
Cellulose	25	-
Cellulose-Soy Flour	20	Soy Flour
Cellulose-Zein	20	Zein
Corn Hull	25	-
Corn Hull-Soy Flour	20	Soy Flour
Corn Hull-Zein	20	Zein
Corn Starch	25	-
Corn Starch-Soy Flour	20	Soy Flour
Corn Starch-Zein	20	Zein
Oat Hulls	25	-
Oat Hulls-Soy Flour	20	Soy Flour
Oat Hulls-Zein	20	Zein
Soy Hulls	25	-
Soy Hulls-Soy Flour	20	Soy Flour
Soy Hulls-Zein	20	Zein

^a Seventy-five percent of each chip consisted of polypropylene.

Table 2. Summary of immobilized cell ethanol fermentations.

Microorganism	Substrate	Max. EtOH Conc./ Productivity	Type of reactor or Special technique	Ref
<i>Saccharomyces cerevisiae</i>	Glucose	190 g/l/h	Mixed-culture biofilm reactor with Oat hull-PP as support material	This study
<i>Saccharomyces cerevisiae</i>	Glucose	40 g/l/h	Biofilm bioreactor with Soy hull-soy flour-PP as support material	This study
<i>Saccharomyces cerevisiae</i>	Sugar cane juice	135 g/l in 8 h	On line removal of toxic end products by high alcohols & activated carbon	32
<i>Saccharomyces cerevisiae</i>	Sugar cane water	4.2-5.3 g/100 ml	Simultaneous extraction and fermentation	27
<i>Saccharomyces uvarum</i>	Non aseptic cane molasses	6.2 g/l/h	CSTR with five stage system for substrate recirculation	7
<i>Zymomonas mobilis</i>	Glucose	13 g/l/h	Batch vertical rotating immobilized cell reactor	2
<i>Zymomonas mobilis</i>	Glucose	63 g/l/h	Continuous vertical rotating immobilized cell reactor	2
<i>Zymomonas mobilis</i>	Glucose	42-46 g/l/h	Cell reactor with trickle flow operation and sponge as packing	23
<i>Zymomonas mobilis</i>	Sucrose	92 g/l/h	Sugar conversion efficiency of 60% with 10% sucrose feed.	26
<i>Zymomonas mobilis</i>	Glucose	364 g/l/h	Biofilm reactor with Soy hull-zein-pp as support	This study
<i>Zymomonas mobilis</i>	Glucose	149.4 g/l/h	Mixed-culture biofilm reactor with Corn starch-soy flour-pp as support	This study

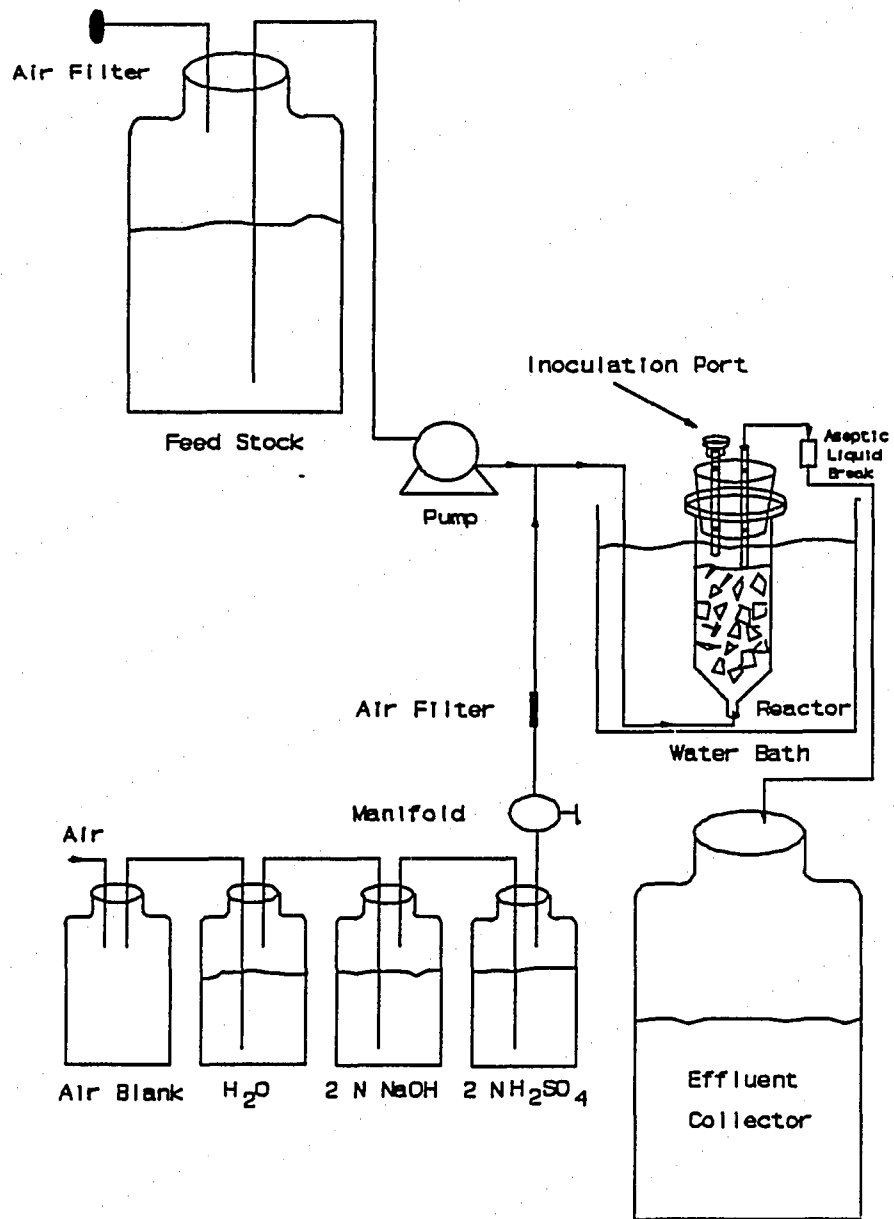


Figure 1. Schematic diagram of the experimental setup of biofilm bioreactor (12).

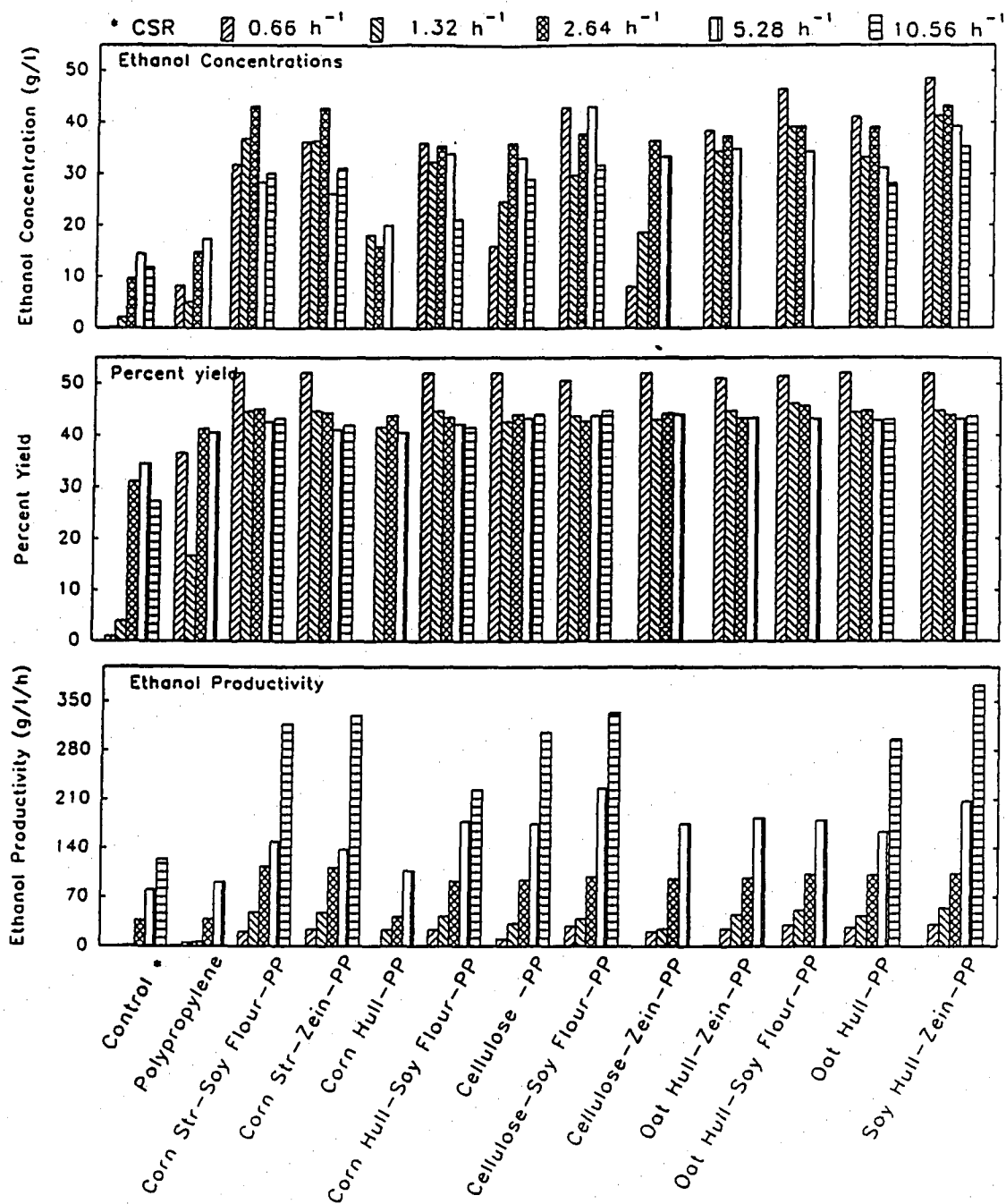


Figure 2. Ethanol concentrations, yield and productivity in pure-culture fermentation with *Z. mobilis* using 10% glucose feed at increasing dilution rates.

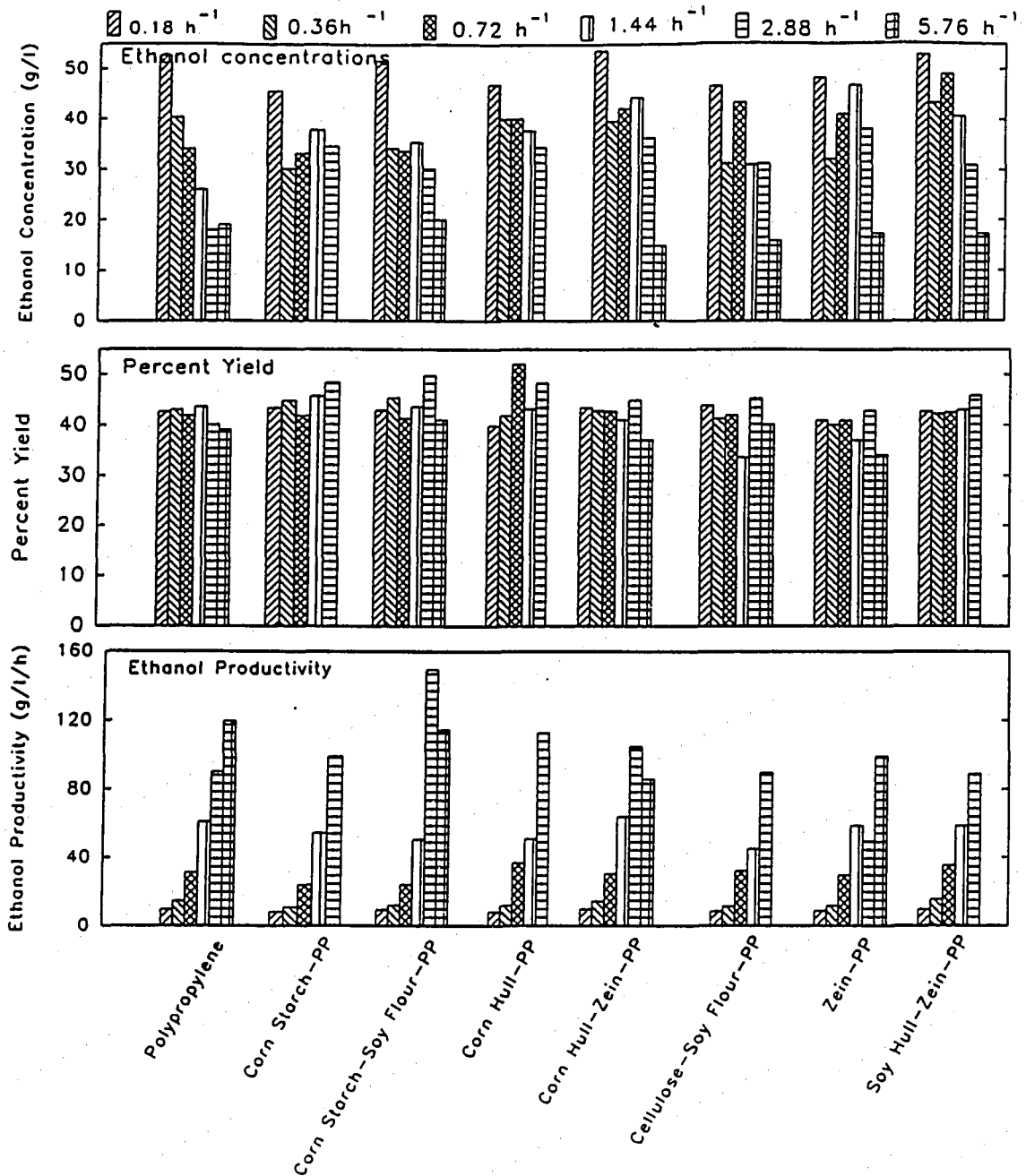


Figure 3. Ethanol concentration, percent yield and productivity in mixed-culture fermentation with *Zymomonas mobilis* and *Streptomyces viridosporus* with 12.5% glucose feed at increasing dilution rates.

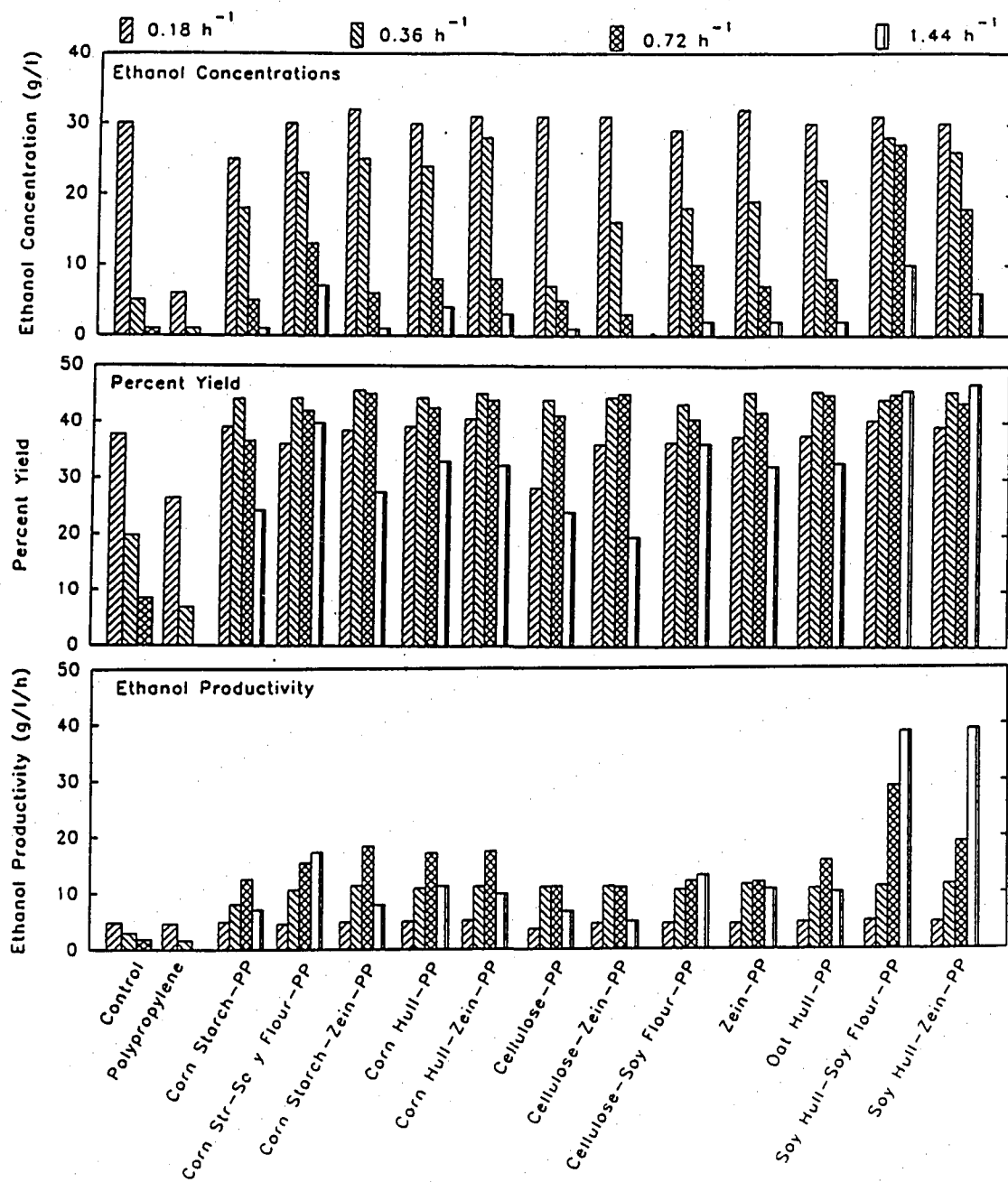


Figure 4. Ethanol concentration, yield and productivity in pure-culture fermentation with *S. cerevisiae* using 7.5% glucose feed at increasing dilution rates.

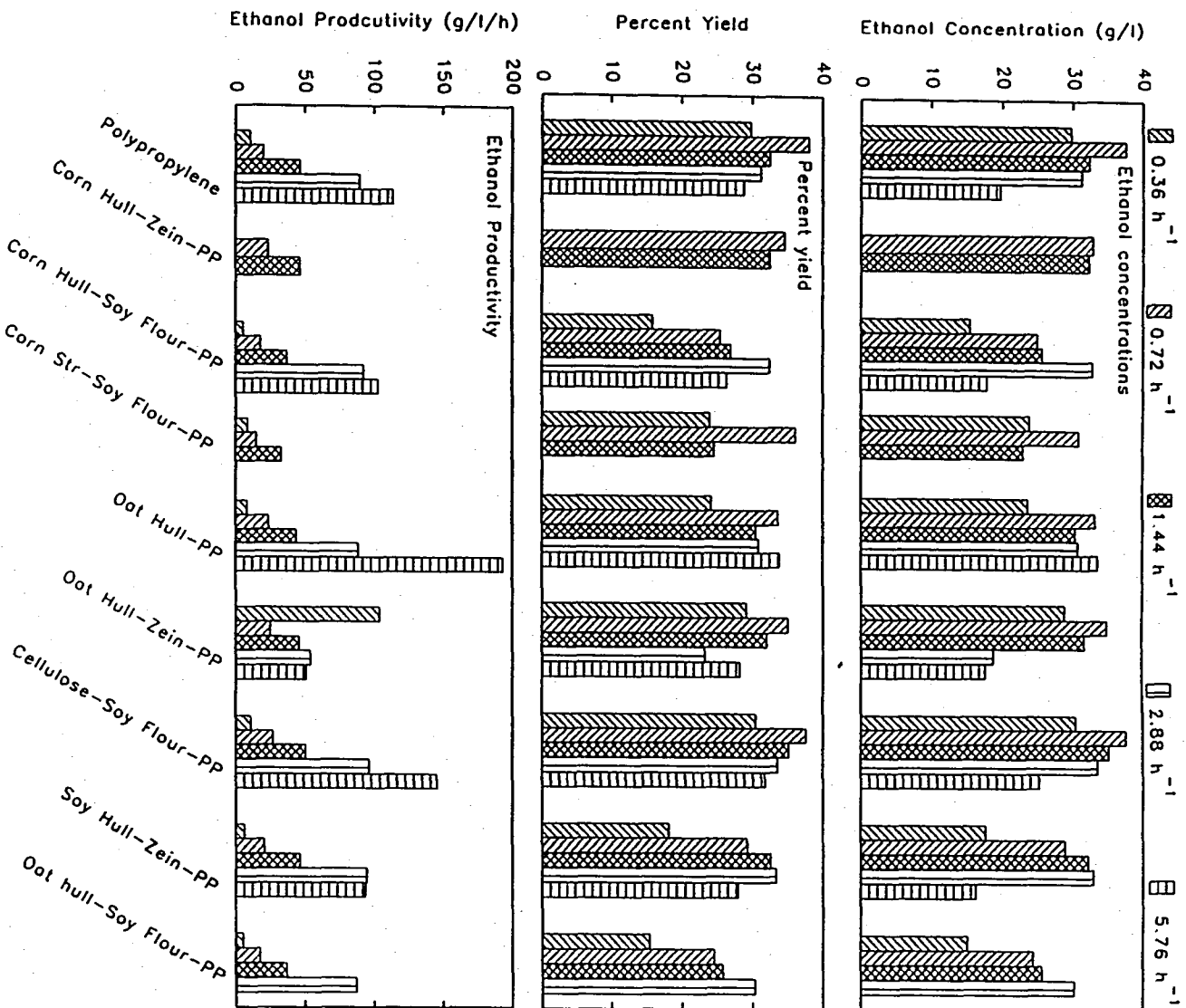


Figure 5. Ethanol concentration, percent yield and productivity in mixed-culture fermentation with *Saccharomyces cerevisiae* and *Streptomyces viridosporus* using 10% glucose feed at increasing dilution rates.

CONTINUOUS ETHANOL PRODUCTION IN BIOFILM REACTORS USING *ZYMOMONAS MOBILIS* AND *SACCHAROMYCES CEREVISIAE*

A paper to be submitted to the Journal of Industrial Microbiology

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Abstract

A 60 day continuous biofilm fermentation was performed with *Zymomonas mobilis* (ATCC 331821) or *Saccharomyces cerevisiae* (ATCC 24859) in reactors with polypropylene or plastic-composite supports. The polypropylene composite supports (2-3 mm chips) used in the reactors contained soybean hulls (20%) and zein (5%) or soybean hulls (20%) and soybean flour (5%) for *Z. mobilis* and *S. cerevisiae*, respectively. A packed-bed reactor that approximated a trickling bed was custom made for *Z. mobilis* fermentation at 30°C by using cylindrical bulb condensers (600 mm long) filled with the plastic composites or polypropylene-alone supports with a 25 ml working volume and a flow from top to bottom. For *S. cerevisiae* fermentation with continuous aeration and 30°C, the reactor columns consisted of cylindrical bulb condensers (400 mm long) filled with plastic composites or polypropylene alone supports with a 30 ml working volume and a flow from bottom to top. Glucose-yeast extract (Ardamine Z,

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Champlain Industries Inc, NJ) medium containing 10% glucose for *Z. mobilis* and 7.5% glucose for *S. cerevisiae* was used. Continuous fermentations in replicates of two were performed for 60 days with dilution rates of 1.92 to 15.36 h⁻¹ for *Z. mobilis*, and 0.18 to 5.76 h⁻¹ for *S. cerevisiae*. Samples were collected every 8 hours and the ethanol concentrations and residual glucose determined by HPLC.

Maximum ethanol productivities of 536 g/l/h with 38.6% yield and 499 g/l/h with 37% yield were obtained with *Z. mobilis* on polypropylene and soybean hull-zein plastic composite supports, respectively. A maximum yield of 50% was observed at a dilution rate of 1.92 h⁻¹ for soybean hull-zein plastic composite supports, whereas with polypropylene-alone supports the maximum yield was 31.5%. A maximum yield of 49% was observed for both polypropylene-alone and soybean hull-zein plastic composite supports at a dilution rate of 3.84 h⁻¹. A dilution rate of 15.36 h⁻¹ produced yields of 35 and 32% on polypropylene and soybean hull-zein plastic composite supports, respectively, with a corresponding reduction in visible biofilm.

With *S. cerevisiae* fermentation the ethanol production was lower with a maximum productivity of 76.1 g/l/h on the plastic composite support. Continuous fermentations with polypropylene alone were discontinued due to excessive back pressure and plugging of the reactor by the cell mass. In cell suspension-culture fermentations maximum productivities of 4.8 and 5.2 g/l/h were obtained with a yield of 24.5 and 25.6% with *S. cerevisiae* and *Z. mobilis*, respectively. Cell washout was observed at a dilution rate of 1.0 h⁻¹.

Introduction

Ethanol can be produced by chemical synthesis from petrochemical feedstocks or by microbial fermentation from renewable plant sources. The microbial production of ethanol was an important process prior to 1940, when chemical synthesis from petrochemical feedstock became more economical. Environmental concerns and possible future depletion of petroleum reserves, however, has revived an interest in ethanol fermentation. The use of ethanol as a fuel for vehicles in the United States has grown to nearly 900 million gallons in 1991 (11). In 1988, 400 million bushels of corn in U.S were utilized for ethanol production adding \$1 billion to farm income (23). Current ethanol production costs by fermentation are less than \$1.25 per gallon depending upon the process used and the feedstock costs.

Denatured alcohol has been used as a solvent for the production of nitrocellulose coatings, shellacs, inks, hydraulic fluids, liquid detergents, soaps, deodorants, perfumes, antiseptics and lotion. Undenatured ethanol is used by the cosmetic, pharmaceutical and food industries in the production of vitamins, flavors and essences, mouthwashes, blood products and fortified wines and as growth substance for single-cell-protein production (13)

Even though ethanol has a wide variety of applications, its high cost is prohibitive. Raw materials are the major costs, representing up to 70% of the final cost. Continued sustainable growth in ethanol utilization depends on improvements in ethanol production and recovery. One approach for improved production is the use of immobilized-cell bioreactors, which retain the biocatalyst (microorganisms) in the

reactor as the substrate and product migrate through.

Several techniques have been applied to improve ethanol fermentation. These include vacuum fermentors (9), cell recycling (22), hollow fiber membrane reactors with recycling (7) and immobilization of cells (3, 5). Supports such as κ -carrageenan gels (12), calcium alginate (15), ion exchange resins (17), vermiculite (2), γ - alumina (16) were used for cell immobilization. Viable cells immobilized in solid gel matrices (i.e. calcium alginate) as beads have been studied in packed-bed and fluidized-bed reactors (25). However, these systems have relatively low efficiency and find limited application due to diffusional resistance of substrate or product and rapid removal of CO_2 from the reactor, together with limited microbial viability for long term production of ethanol. Improving industrial fermentation productivity requires development of increased production rates with reduced fermentor volumes and decreased operating costs.

Biofilms are a natural form of cell immobilization that result from microbial attachment to solid supports (4). Biofilms have been used in waste water treatment plants (18), for production of vinegar by the "quick vinegar process", mineral ore treatment (8) and ethanol production. This paper describes the use of biofilm reactors with plastic composite supports for enhanced ethanol production. Ethanol productivities 10 to 100 times greater than those in suspension culture were obtained in the biofilm reactors. *Zymomonas mobilis* had higher productivities than *Saccharomyces cerevisiae*. A maximum productivity of 536 g/l/h with 38.6 % yield was obtained with *Z. mobilis* using polypropylene supports, which is the highest reported to date.

Materials and Methods

Microorganisms and media. *Zymomonas mobilis* (ATCC 31821) was maintained in a medium containing 2% (w/v) glucose, 0.5% (w/v) yeast extract (Difco Laboratories, Detroit, MI), 0.2% (w.v) $(\text{NH}_4)_2\text{SO}_4$, 0.05% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2% (w/v) KH_2PO_4 at 4°C and was subcultured every two weeks. *Saccharomyces cerevisiae* (ATCC 24859) was maintained on a medium containing 2% (w/v) glucose, 1.0% (w/v) yeast extract (Difco) and 2% (w/v) peptone at 4°C and subcultured every 4-6 weeks.

For *Z. mobilis* the fermentation medium consisted of 0.5% (w/v) yeast extract (Ardamine Z, Champlain Industries, Clifton, NJ), 0.2% (w/v) $(\text{NH}_4)_2\text{SO}_4$, 0.05% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2% (w/v) KH_2PO_4 and 10% (w/v) glucose (pH 5.8). Medium used for *S. cerevisiae* fermentations contained 0.6% (w/v) yeast extract (Ardamine Z), 0.023% (w/v) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15% (w/v) KH_2PO_4 , 0.4% (w/v) $(\text{NH}_4)_2\text{SO}_4$ and 7.5% glucose (pH 5.8). Sixty liters of fermentation media were sterilized at 121°C for 15 min in a 72 L B Braun U50 fermentor (B. Braun, Allentown, PA) and transferred aseptically to pre-sterilized 50 L carboys. The phosphate salts were autoclaved separately and added to the medium aseptically after sterilization and prior to dispensing into carboys.

Support materials. Polypropylene composite chips containing agricultural materials (25% w/w) were used as solid supports. The plastic composite supports were prepared by high-temperature extrusion of the polypropylene (Quantum USI Division, Columbus, OH.) and agricultural materials in a Brabender PL2000 counter-rotating twin-screw

extruder (C. W. Brabender Instruments, Inc., South Hackensack, N.J) by using the method of Demirci *et al* (10). The barrel temperatures were 200, 210 and 220°C, the die temperature was 220°C and the screw speed was 20 rpm. The agricultural products used were soybean flour (Archer Daniel Midland Co., Decatur, IL), ground soybean hulls (Iowa State University Center for Crops Utilization Research) and zein (Sigma Chemical Co., St. Louis, MO). Each agricultural material was vacuum dried for 48 h at 110°C prior to being used for extrusion. Polypropylene pellets and specific agricultural blends were mixed for several minutes in a container and then added to the extruder hopper. Polypropylene was compounded with 20% soybean hull and 5% zein, or 20% soybean hull and 5% soybean flour, and extruded as 3-mm diameter rods, air cooled and then cut into chips of 2-3 mm in length with a pelletizer.

Bioreactors. A packed-bed reactor that approximated a trickling bed was custom made for *Z. mobilis* fermentation at 30°C. The reactor consisted of cylindrical bulb condenser (600 mm long, Corning part No. 2420-600) filled with 140 ml (65 g) of soybean hull-zein plastic composites or polypropylene-alone supports with a 25 ml working volume and a flow from top to bottom (Figure 1). A constant volume of 25 ml was maintained by adjusting the height of the liquid break in the exit line. The lower and upper ends of the condenser were fitted with 3 ml syringe plungers with the top pad perforated with holes to retain the support materials within the reactor. Liquid breaks were used both in the feed and exit lines to prevent contamination when drawing samples or switching carboys. A rubber septum in the stopper on the liquid break in the feed inlet was used

for inoculating the microorganism into the reactor.

A plug-flow bioreactor was custom made for *S. cerevisiae* fermentation with continuous aeration and 30°C. The reactor column consisted of cylindrical bulb condenser (400 mm long, Corning part No. 2420-400) filled with the 65 ml (18.5 g) of soybean hull-soybean flour plastic composites or polypropylene-alone supports with a working volume of 30 ml. The reactor was built similar in construction to the one described for *Z. mobilis*, but with the feed inlet from the bottom of the reactor (Figure 2). Filter sterilized air was supplied to the reactors with *S. cerevisiae*. Continuous fermentations in replicates of two were carried out for 60 days with dilution rates of 1.92 to 15.36 h⁻¹ for *Z. mobilis* and 0.18 to 5.76 h⁻¹ for *S. cerevisiae* in replicates of two.

A continuously stirred tank reactor (CSTR) (2 L Biostat M, B. Braun) with agitation at 250 rpm and a 300 ml working volume was used as a control. A Y-connector was placed on the exit line with one arm of the Y-connector connected to the effluent exit line and the other arm left open in the reactor. The height of the Y-connector was adjusted to maintain a constant volume of 300 ml in the reactor. For *Z. mobilis* nitrogen gas was continuously supplied (160 ml/min) to the suspension reactors to maintain an anaerobic environment. For the *S. cerevisiae* CSTR, air was continuously supplied (160 ml/min). Liquid breaks in the feed inlet and exit line were used to prevent contamination during sampling. The fermentors were operated at 30°C with dilution rates of 0.5 h⁻¹ and 1.0 h⁻¹ for a week.

Continuous fermentation. Each reactor (packed bed or CSTR) was inoculated with 1% of the specific 24 h culture and incubated in batch fermentation at 30°C for 24 hours, then changed to continuous fermentation with various dilution rates. Samples were collected at 8 hour intervals from the exit line and analyzed for cell density, ethanol and glucose concentration. Dilution rates were routinely confirmed by measuring the exit volumes.

Evaluation of the biofilm. The biofilm formed on the support material was evaluated visually by the accumulation of the cell mass on the chips and by Gram staining. Gram staining was performed on the supports after the fermentation and the resulting color development was compared visually with the color of the uninoculated Gram stained supports. Yeast cell stained violet. Weight increase of the supports was not determined due to the difficulty in removing the supports from the reactor.

Analysis of culture broth. The suspended cell density in the reactors was measured by absorbance at 620 nm using a Bausch and Lomb Spectronic 20 spectrophotometer (Milton Roy, Rochester, NY). Percentage glucose and ethanol were measured by using a Waters High Pressure Liquid Chromatograph (Millipore Corporation, Milford, Ma.) equipped with Waters Model 401 refractive index detector, column heater, autosampler and computer controller. The separation of ethanol, glucose and other broth ingredients were done on a Bio-Rad Aminex HPX-8711 column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, Ca.) using 0.012 N Sulfuric acid as a mobile phase at a

flow rate of 0.8 ml/min with a 20 μ l injection volume and a column temperature of 65°C.

Residence time. The residence time of the medium in the reactors was determined by injecting 1 ml of 1% aqueous solution of dextran blue dye (Sigma Chemical Co., St. Louis, MO) into the feed line of the fermentation medium. Flow rate was determined by collecting 10 ml samples until all the dye eluted from the reactor. The residence time was calculated by the fraction that had the highest intensity of color as observed by absorbance at 540 nm.

Results and Discussion

Continuous fermentation. For *Z. mobilis* the reactors were operated at each dilution rate for a week until it achieved a dilution rate of 15.36 h⁻¹. At this highest dilution rate the reactor was operated for 5 days as there was a visible decrease in biofilm and in ethanol production (figure 3). It was also difficult to maintain this high dilution rate because of the large consumption of media. There was 10.7 g/l of glucose and 34.5 g/l ethanol in the effluent with a 10% glucose feed. Thereafter the fermentation was continued at a dilution rate of 7.68 h⁻¹ for another 30 days. With *S. cerevisiae*, the reactors were operated for one week at each of the dilution rates. At a dilution rate of 5.6 h⁻¹ a decrease in the visible biofilm and an overall reduction in ethanol production (figure 4) was observed. There was 30.4 g/l of glucose and 13 g/l of ethanol in the effluent with a 7.5% glucose feed. Therefore, the reactors were operated at a dilution

rate of 2.8 h^{-1} for the next 30 days. The ethanol concentrations and productivities obtained in the biofilm reactors operated for long term, were similar, to the ethanol concentrations and productivities observed at the corresponding dilution rates with both *Z. mobilis* and *S. cerevisiae*. The values of the replicates were within an average of 5%.

Percent Yield. Percent yield is a measure of the conversion efficiency of glucose to ethanol and is defined as ethanol produced divided by glucose consumed. Theoretical yield for ethanol production is 51% (29).

In suspension-culture reactors the yield was 25.6% with *Z. mobilis* and in the biofilm reactor the yields were 31.5 to 49% with polypropylene-alone and 37 to 51% with soybean hull-zein-plastic composite supports (Figure 3). The percent yields were lower on polypropylene composites during the first few days of fermentation but were comparable by the end of first week of fermentation. With *Z. mobilis* fermentation yields of 38.6 and 37% were obtained at a dilution rate of 15.36 h^{-1} with polypropylene alone and soybean hull-zein plastic composite supports, respectively. With *S. cerevisiae* using soybean hull-soybean flour-plastic composite supports, the percent yield were 29 and 43% at dilution rates of 5.76 h^{-1} and 2.88 h^{-1} , respectively. A 24.5% yield was obtained with suspension culture fermentation of *S. cerevisiae* at a dilution rate of 0.5 h^{-1} (Figure 4).

Ethanol productivity. Productivity (g/l/h) is a measure of ethanol production per hour (calculated as ethanol produced in g/l times the dilution rate in h^{-1}). Ethanol productivities were low in suspension-culture fermentations for both *Z. mobilis* and *S.*

cerevisiae which were 5.25 and 4.85 g/l/h, respectively. Ethanol productivities of 76.1 and 39.7 g/l/h were obtained with *S. cerevisiae* on soybean hull-soybean flour plastic composite supports at dilution rates of 2.88 and 1.44 h⁻¹, respectively (Figure 4). At a dilution rate of 5.76 h⁻¹ an ethanol productivity of 72.8 g/l/h was obtained with 29% yield. Fermentation with *S. cerevisiae* in polypropylene alone reactors was not successful as there was plugging of the reactors due to excess cell mass. Maximum productivity achieved in CSTR suspension-culture fermentations was 4.8 g/l/h for *S. cerevisiae*. A complete washout was observed when the suspension culture fermentation was carried out at a dilution rate of 1.0 h⁻¹. A maximum productivity of 536 and 499 g/l/h was observed on polypropylene and soybean hull-zein plastic composite supports with *Z. mobilis* fermentation, respectively, at a dilution rate of 15.36 h⁻¹. These ethanol productivities are significantly higher than those reported in the literature (Table 1).

Ethanol production. Ethanol and glucose concentrations were analyzed from samples collected every 8 h to determine the steady-state condition. A steady state condition was usually observed within 24 h of continuous fermentations at each of the dilution rates tested. The ethanol concentrations were consistently higher for plastic composite support reactors for both *Z. mobilis* and *S. cerevisiae* than for cell suspension reactors (Figure 3 and 4). The cell densities from the effluents of the plastic-composite-supports bioreactors were higher than the cell densities from the effluents of cell suspension CSTR (Figure 5). Continued ethanol production with corresponding increase in cell mass in the effluent at the dilution rates above the CSTR washout confirmed biofilm

formation. At these faster dilution rates suspended cell concentration increase or cell loss were linked with reduced yields. In the biofilm reactors, a decrease in the visible biofilm was observed at dilution rates of 15.36 h^{-1} for *Z. mobilis* and 5.76 h^{-1} for *S. cerevisiae*. The ethanol concentrations were higher in the reactor with soybean hull-zein plastic composite support in the initial stages of *Z. mobilis* fermentation. However, within a week there was no appreciable difference in the ethanol concentrations between the plastic composite support and the polypropylene-alone support bioreactor. The visual appearance of biofilm formation on the polypropylene alone supports in the initial stages of fermentation was very low which corresponded with the low cell densities in the effluent from those reactors. The protein and amino acids in the soybean hull and zein of the plastic composite support potentially provides the micronutrient for *Z. mobilis* and provides a unique surface for biofilm formation. However, once cell mass started to accumulate in the trickle flow reactor there was no difference in the performance of the two supports. Highest ethanol concentrations of 50 g/l were obtained with *Z. mobilis* fermentation using soybean hull-zein plastic composite support at a dilution rate of 1.82 h^{-1} . At the highest dilution rate tested (15.36 h^{-1}), the ethanol concentrations were 32.5 and 34.5 g/l in soybean hull-zein plastic composite and polypropylene-alone reactors, respectively. At the end of 60 days of fermentation the mean residence time of the medium was 7 min at a dilution rate of 7.60 h^{-1} . Maximum ethanol concentrations of 10.5 g/l was achieved in suspension culture fermentation of *Z. mobilis* from 10 g/l glucose feed.

With *S. cerevisiae* the ethanol concentrations obtained were 28.4 , 27.2 and 13

g/l at dilution rates of 1.44, 2.88 and 5.76 h⁻¹, respectively, on soybean hull-soybean flour plastic composite support reactors. The mean residence times of the medium were 29 and 18 min at dilution rates of 1.44 and 2.88 h⁻¹, respectively. Eventhough the residence time of the medium was longer in the biofilm reactors with *S. cerevisiae* the ethanol productivities obtained were lower than the biofilm reactors with *Z. mobilis*. The ability to form better biofilms together with higher glucose uptake rates of *Zymomonas* resulted in higher productivities. The mean residence time was 7 min at a dilution rate of 7.68 h⁻¹ for *Z. mobilis*. A maximum ethanol concentration of 9.7 g/l was observed in suspension-culture fermentations at dilution rate of 0.5 h⁻¹

Support materials and bioreactor design. Soybean hull-zein plastic composite supports demonstrated high concentrations of ethanol and good retention of biomass in the early stages of fermentation with *Z. mobilis*. This suggests that the soybean hull and zein in the composite support is providing some micronutrient (proteins and amino acids) to *Z. mobilis* and that the plastic composite support is providing a better support for cell attachment. However, as this down flow fermentation progressed there was no appreciable difference in the ethanol concentrations between the two supports. We attribute this to the reactor and support design which stimulated cell retention. The packed bed design which resulted from the plastic support shape (2-3 mm chips) increased the back pressure and decreased the flow rate at which medium was pumped into the reactor. Therefore, the actual flow rate was monitored daily. Adjustments were made, whenever needed, to the flow rate to maintain the desired dilution rate.

The *S. cerevisiae* fermentation in the polypropylene-alone upflow reactors plugged and resulted in excessive back pressure due to cell mass accumulation on the plastic support. The plugging of the bioreactor was due to the flocculating characteristics of the yeast and not because of the formation of the biofilm as noticed by the flocculation of the cells and compacting the support material without much biofilm formation. In the preliminary studies, this phenomenon was not observed. This may be because the flow rates were changed everyday and there was not enough time for the yeast to flocculate in the bioreactor. The plastic composite support with the yeast remained operational for 60 days, which suggests that the agricultural material imparted some control of biofilm thickness. Support shape did contribute to bioreactor plugging. Therefore, different support shapes such as Raschig rings need to be evaluated before scale-up of this process.

Conclusions

The use of biofilm reactors for enhanced ethanol production in continuous fermentation was demonstrated. The use of plastic composite supports increased ethanol productivities for *Z. mobilis* and *S. cerevisiae* fermentation. The use of *Z. mobilis* in biofilm reactors with the composite material is suggested for continuous fermentation to improve ethanol productivities. The flocculating characteristic of the yeast rendered the bioreactor containing polypropylene supports inoperative. A further

change in plastic composite support will reduce the pressure build up and plugging of the bioreactor. In 60-day continuous fermentation dilution rates 3- and 4- fold higher than current CSTR fermentation can be achieved with only a minor reduction in percent yield.

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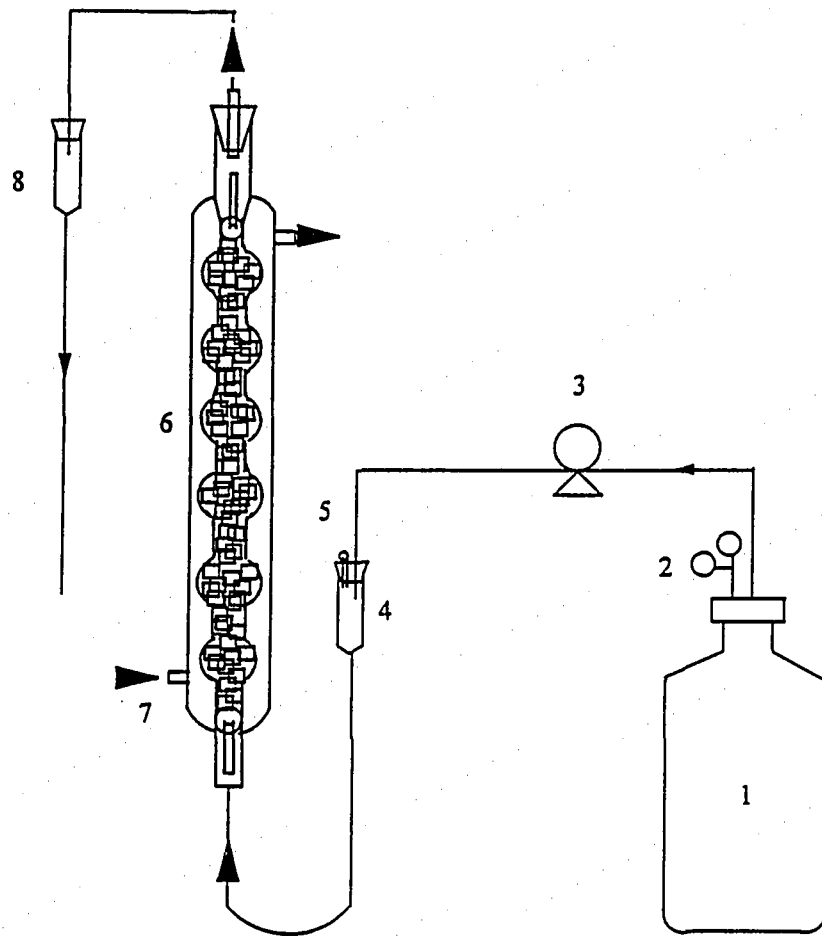
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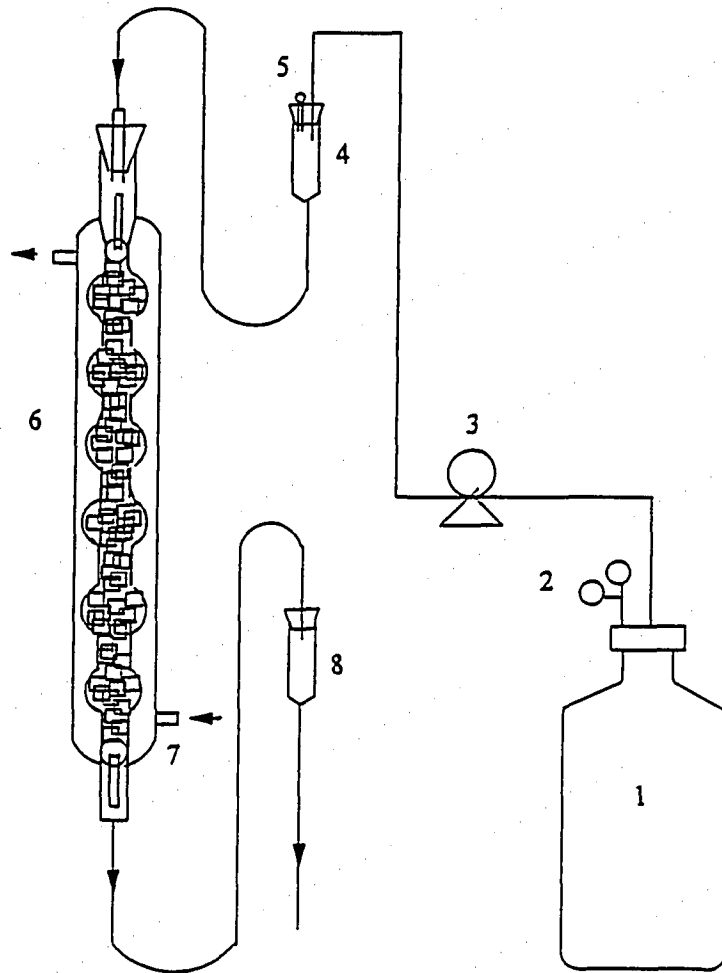
Table 1. Summary of immobilized cell ethanol fermentation

Microorganism	Substrate	Max. EtOH Conc./ Productivity	Type of reactor or Special technique	Ref
<i>Saccharomyces cerevisiae</i>	Glucose	76.1 g/l/h	Biofilm bioreactors with Soy hull-soy flour plastic composite support	This study
<i>Zymomonas mobilis</i>	Glucose	536 g/l/h	Biofilm bioreactor with Soy hull-zein-PP supports	This study
<i>Saccharomyces cerevisiae</i>	Sugar cane juice	135 g/l in 8 h	On line removal end products by high alcohols & activated carbon	25
<i>Saccharomyces cerevisiae</i>	Sugar cane water suspension	4.2-5.3 g/100 ml	Simultaneous extraction and fermentation	22
<i>Saccharomyces uvarum</i>	Non aseptic cane molasses	6.2 g/l/h	Continuously stirred tank reactor with five stage system for substrate recirculation	6
<i>Zymomonas mobilis</i>	Glucose	13 g/l/h	Batch vertical rotating immobilized cell reactor	1
<i>Zymomonas mobilis</i>	Glucose	63 g/l/h	Continuous vertical rotating immobilized cell reactor	1
<i>Zymomonas mobilis</i>	Glucose	42-46 g/l/h	Cell reactor separators with trickle flow operation and sponge as packing	19
<i>Zymomonas mobilis</i>	Sucrose	92 g/l/h	Sugar conversion efficiency of 60% with 10% sucrose feed. Culture isolated from sugarcane juice	22



- | | | | |
|---------------------|-----------------------------|--------------------------|---------------------|
| 1. Feed reservoir | 2. Air filters | 3. Pump | 4, 8. Liquid breaks |
| 5. Inoculation port | 6. Bioreactor with supports | 7. Water jacketed column | |

Figure 1. Schematic diagram of an upflow biofilm bioreactor used for continuous fermentation with *Z. mobilis*.



- | | | | |
|---------------------|-----------------------------|--------------------------|---------------------|
| 1. Feed reservoir | 2. Air filters | 3. Pump | 4, 8. Liquid breaks |
| 5. Inoculation port | 6. Bioreactor with supports | 7. Water jacketed column | |

Figure 2. Schematic diagram of a downflow biofilm bioreactor used for continuous fermentation with *S. cerevisiae*.

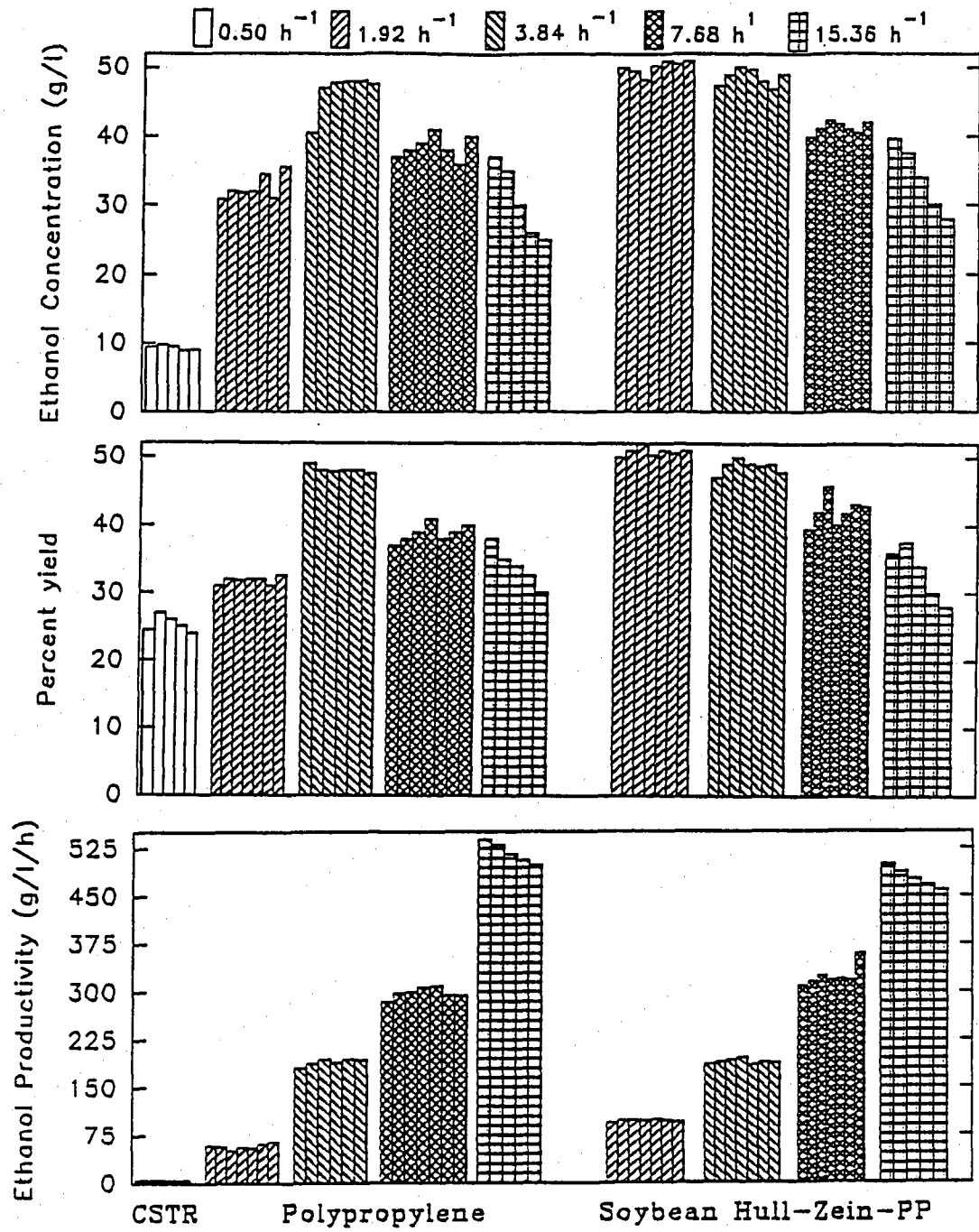


Figure 3. Ethanol concentrations, yield and productivities in downflow biofilm bioreactors used for continuous fermentation with *Z. mobilis* (each data point represents the average value of two replicate data points taken every day. The fermentors were operated at each dilution rate for 5-7 days and increased sequentially).

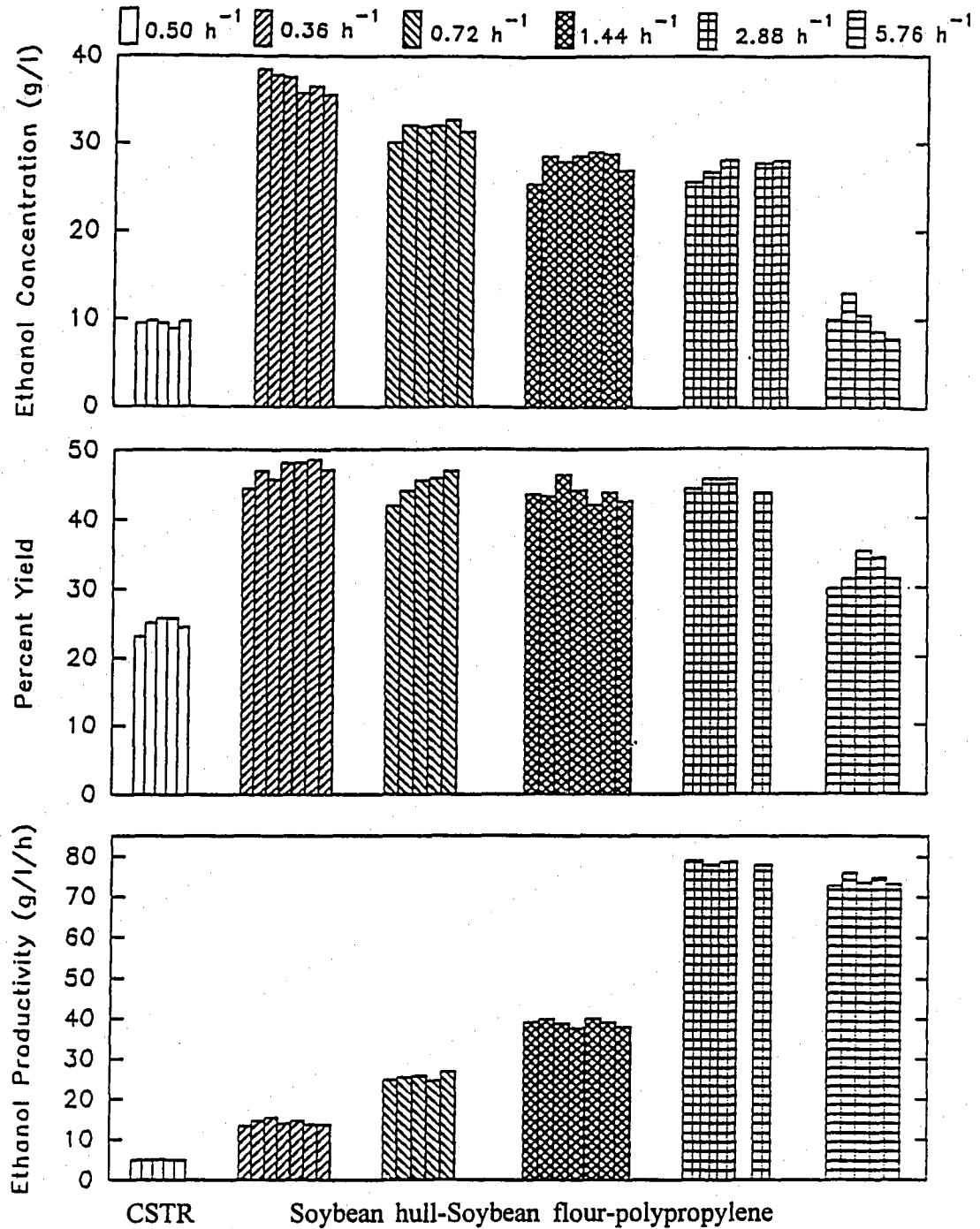


figure 4. Ethanol concentrations, yield and productivities in upflow biofilm bioreactors used for continuous fermentation by *S. cerevisiae* (each bar represents the average of two replicate data points taken every day. The fermentors were operated at each dilution rate for 5-7 days and increased sequentially).

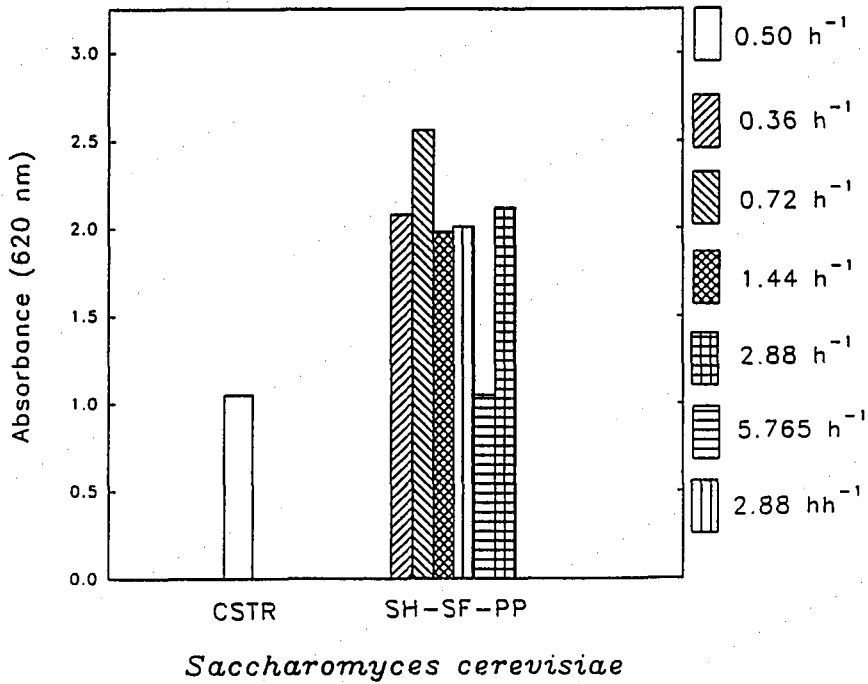
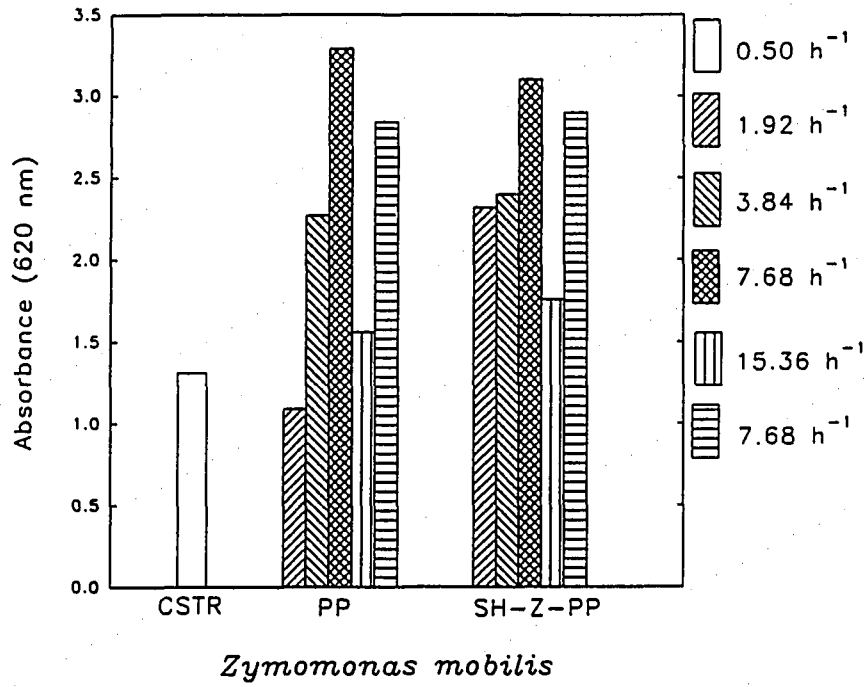


Figure 5. Absorbance (at 620 nm) of effluents from continuous fermentation reactors operated at different dilution rates (average of two replicates).

SUMMARY AND CONCLUSIONS

Summary. The economic success of the production of fermentation ethanol depends on lowering the costs of production. This can be achieved by reducing the capital costs and by improving the fermentation processes by development of a simple and inexpensive continuous fermentation process.

Plastic composite supports were prepared by temperature extrusion of polypropylene and up to 25% (w/w) various agricultural materials into 2-3 mm chips. Pure cultures of *Zymomonas mobilis* or *Saccharomyces cerevisiae* and mixed-cultures with either ethanol-producing microorganism and the biofilm forming *Streptomyces viridosporus* T7A were evaluated in continuous fermentations at dilution rates of 0.18 to 10.56 h⁻¹. A maximum ethanol productivity of 374 g/l/h with 44% yield was obtained using soybean hull-zein plastic composite supports with *Z. mobilis* and a 10% glucose feed. Mixed-culture fermentations with *Z. mobilis* and *S. viridosporus* resulted in lower yields and decreased productivities. Fermentations with *S. cerevisiae* in pure-culture resulted in a maximum ethanol productivity of 40 g/l/h with 47% yield on soybean hull-soybean flour plastic composite supports. The maximum productivity of 190 g/l/h with 35% yield was obtained in mixed-culture fermentations of yeast and streptomycete. The use of soybean flour, soybean hull and zein in the composite support provided micronutrients (proteins and aminoacids) to the microorganisms and resulted in better biofilm formation and increased productivities.

In long term fermentation studies using a down flow biofilm reactor for *Z. mobilis*, the formation of biofilm was more on the plastic composite support during

the first week of fermentation. This suggests that the composite support provided micronutrients to the microorganism and enabled it to form a biofilm. However, there was no appreciable difference in the performance of the polypropylene and the plastic composite support over a longer period of time. This is because *Z. mobilis* is a good biofilm former. Maximum ethanol productivities of 536 g/l/h and 499 g/l/h were obtained with *Z. mobilis* using polypropylene alone and soybean hull-zein composite support, respectively. With yeast fermentations a maximum productivity of 78.4 g/l/h was obtained on soybean hull-soybean flour composite support. Reactors with polypropylene alone could not be operated with *S. cerevisiae* due to support shape and the excessive build up of the cell mass caused by the flocculating characteristic of the yeast. Visible biofilm formation was observed in all bioreactors within two weeks of operation. Suspension-culture fermentations resulted in ethanol productivities of 4.5 and 5.2 g/l/h with yeast and bacteria, respectively. A cell washout was observed in suspension culture fermentation when operated at a dilution rate of 1.0 h^{-1} . A change in the shape of the support needs to be evaluated to further improve ethanol fermentation in biofilm reactors.

Conclusions.

1. Improved ethanol production can be achieved by using the plastic composite supports in biofilm bioreactors.
2. *Z. mobilis* is the preferred organism for use in biofilm bioreactors because of its excellent biofilm forming capability.

3. Mixed-culture fermentations resulted in decreased yields and productivities with *Z. mobilis* but improved the productivities with *S. cerevisiae* but with a reduced yield. Hence, mixed-culture fermentations are not suitable for use in biofilm reactors.
4. The use of soybean hull, soybean flour and zein in the composite supports provided micronutrients to the microorganisms and resulted in the formation of better biofilm and increased productivities.
5. A 10-100 fold increase in productivities were obtained in continuous fermentation with the plastic composite support when compared to the cell suspension culture fermentations with *Z. mobilis*.
6. The use of polypropylene alone is not preferred with *S. cerevisiae* fermentations because of the potential of plugging of the bioreactor.
7. A change in the shape of the support needs to be evaluated to further improve the ethanol production in biofilm reactors.

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