Comparison of Different Production Processes for Bioethanol

Belkıs ÇAYLAK

Ege University, Ege Technical Highschool
Bornova, İzmir - TURKEY

Fazilet VARDAR SUKAN

Ege University, Faculty of Chemistry Engineering
Bornova, İzmir - TURKEY

In this study, ethanol was produced with the microorganism *Saccharomyces cerevisiae* by using sucrose as a substrate. Batch processes were tested by using the same substrate, microorganism and medium composition. *Saccharomyces cerevisiae*, used in the free form in the first group of experiments, was immobilized by different methods. In the second group of experiments agar, sponge and a natural material called luffa cylindrica fiber were used as support materials. The batch processes with free and immobilized microorganisms were compared with respect to efficiency and yield.

Introduction

The decreasing reserves and increasing value of petrochemicals have renewed the interest in the production of bioethanol and its use as fuel and chemical feedstock.

Ethanol can be produced from many different raw materials, which are grouped according to the type of carbohydrates they contain, i.e., sugar, starch or cellulose. Sugar for ethanol production (sucrose, glucose or fructose) may be derived from any of the three classes of raw materials[1]. Industrial processes for the production of ethanol by fermentation of molasses, beet, cane or grain sugars are well established. Since sugars are already available in a degradable form and yeast cells can metabolize sugars directly, these substrates require the least costly preparation. The other carbohydrates must be hydrolyzed to sugars before they can be metabolized. So, although starchy or cellulosic materials are cheaper than sugar-containing raw materials, the requirement of converting the starch or cellulosic materials to fermentable sugars is a disadvantage of these substrates[2].

Ethanol can be produced by four main types of industrial operations: batch, continuous, fed-batch and semi-continuous[3].

In batch fermentation, substrate and yeast culture are charged into the bioreactor together with nutrients[3]. Most of the ethanol produced today is done by the batch operation since the investment costs are low, do not require much control and can be accomplished with unskilled labor. Complete sterilization and management of feedstocks are easier than in the other processes. The other advantage of batch operation

is the greater flexibility that can be achieved by using a bioreactor for various product specifications.

In the continuous process, feed, which contains substrate, culture medium and other required nutrients, is pumped continuously into an agitated vessel where the microorganisms are active. The product, which is taken from the top of the bioreactor, contains ethanol, cells, and residual sugar[4].

The fed-batch operation, which may be regarded as a combination of the batch and continuous operations, is very popular in the ethanol industry. In this operation, the feed solution, which contains substrate, yeast culture and the required minerals and vitamins, are fed at constant intervals while effluent is removed discontinuously. The main advantage of the fed-batch system is that inhibition and catabolite repression are prevented by intermittent feeding of the substrate. If the substrate has an inhibitory effect, intermittent addition improves the productivity of the fermentation by maintaining a low substrate concentration. It is essential to keep the culture volume constant in continuous operation, whereas there is volume variation in the fed-batch processes.

In semi-continuous processes, a portion of the culture is withdrawn at intervals and fresh medium is added to the system. In the continuous processes it is essential to maintain a constant culture volume, whereas there is volume variation in semi-continuous processes. This method has some of the advantages of the continuous and batch operations. There is no need for a separate inoculum vessel, except at the initial start-up. Time is also not wasted in non-productive idle time for cleaning and resterilization. Another advantage of this operation is that not much control is required. However, there is a high risk of contamination and mutation due to long cultivation periods and periodic handling. Furthermore, since larger reactor volumes are needed, slightly higher investment costs are required.

Ethanol production from biomass by fermentation is possible by using free or immobilized cells. Both have advantages and disadvantages. Microorganisms used in industry are selected to provide the best possible combination of characteristics for the process and equipment being used. The selected strains should have tolerance to high concentrations of sugar and alcohol[3]. The use of immobilized whole cells in industrial processes has attracted considerable attention during the past few years due to advantages over traditional processes[5]. Immobilization is the restriction of cell mobility within a defined space. Immobilization provides high cell concentrations and cell reuse. It also eliminates washout problems at high dilution rates and the costly processes of cell recovery and cell recycle. High volumetric productivities can also be obtained with the combination of high cell concentrations and high flow rates. Immobilization may also improve genetic stability.

The aim of this study was to compare different modes of operations with respect to productivity by using glucose as a substrate and free and immobilized *Saccharomyces cerevisiae* as catalyzers. Batch, continuous, fed-batch and semi-continuous operational modes were studied under laboratory conditions.

Materials and Methods

A growth medium tank, a peristaltic pump, a fermenter, an air pump and a product tank were used as materials.

Microorganism: All fermentations were carried out using *Saccharomyces cerevisiae*, a strain of commercial baker's yeast obtained from Pakmaya and kept in the yeast culture collection of the Department of Food Engineering, Faculty of Engineering, Ege University.

Fermentation Medium: One liter of production medium was prepared according to the requirements of *Saccharomyces cerevisiae*, containing 22 % sucrose, 0.3 % dry yeast extract, 0.5 % peptone, 0.15 % $(NH_4)_2$ HPO_4 and 0.3 % $MgSO_4$ in tap water.

Inoculum Preparation: Dry baker's yeast was used directly in all experiments except those with immobilized cells. In the fermentations with free cells, 50 ml of stock culture solution containing the given amounts of nutrients was transferred into a 250 ml Erlenmeyer flask, the pH was adjusted to pH 5.0 with 0.1 N phosphoric acid and the solution was autoclaved at $121°C$ for 20 min. 0.5 g of dry baker's yeast was added after sterilization. The mixture was then incubated at $28°C$ on an Infors AG-CH-4103 Bottmingen-type shaker working at 200 rpm for a period of 4 hours before inoculation into the production vessel.

In the fermentations with immobilized cells, 2% agar solution was used as the immobilization material for the physical entrapment method. 100 ml of agar solution and 50 ml of yeast culture were prepared separately. Inoculum preparation was the same as before.

Sponge cubes and luffa cylindrica fibers were used as the support materials for immobilization by adsorption. 200 ml of stock culture containing the nutrients was used for inoculation. The culture was then divided to five 250 ml Erlenmeyer flasks in order to provide good mixing for immobilization. Sponge cubess were added to these flasks in equal amounts and sterilized at $121°C$ for 20 min. 0.5 g of dry dry baker's yeast was then added and the flasks were placed on a rotary shaker for 4 hr at 200 rpm and $28°C$ prior to inoculation of the fermenter. The same method was used for immobilization on luffa cylindrica fiber surfaces.

Ethanol Fermentation with Free Cells: In batch fermentation, 400 ml fermentation medium was inoculated with 50 ml inoculum and the pH was adjusted to pH 5.0. Fermentation was carried out in a rotary shaker at 200 rpm and $28°C$. The weight decrease was measured every 2 hours in order to determine the amount of ethanol. Fermentation was terminated after 96 hours.

In continuous fermentation, 150ml medium was inoculated with 50 ml inoculum. The system was cultivated aerobically for 15 hours. Consequently, 150 ml of growth medium was added to the fermenter and run for 7 hours under anaerobic conditions to obtain a certain amount of cell concentration and product. At the end of the preliminary batch period, continuous feeding containing 12% sucrose was initiated at a flowrate of 36 ml/hr. The weight decrease of the system was measured every 5 hours for 2 weeks.

In the fed-batch process, a concentrated feed solution was prepared in order to keep the volume change of the reactor at a minimum. Since maintaining a constant substrate concentration throughout the process is the most crucial parameter for this operational mode, substrate additions were at equal intervals and amounts. It was found that at $25°C$, the solubility of sucrose is 90.9 g in 100 ml of water[8]. From batch operation data it was determined that microorganisms utilized 6 g of sucrose in 8 hours. Twenty-seven tubes containing 6.6 ml concentrated sucrose solution were prepared and the pH was adjusted to pH 5.0. The substrate concentration in the reactor was 10% at the beginning of the fermentation. The concentrations of mineral salts were 3 times those used in batch fermentation since the fermentation time was 3 times longer. The initial substrate was consumed after one day, and subsequently additions were begun. A total of twenty-seven additions every 8 hours were made during the process. The weight decrease of the system was measured every 2 hours for ten days.

In the semi-continuous fermentation, the start-up of the process was the same as with the batch operation. Following a growth of 72 hours, 2/3 of the reactor contents (200) ml was removed and 200 ml of fresh growth medium was added by peristaltic pump. During the 10 days of total cultivation this process was repeated every 2 days.

The time course of ethanol production, sucrose consumption and volumetric productivity was determined for each cultivation process.

Ethanol Production with Immobilized Cells: Batch fermentation was repeated with immobilized cells in order to examine the advantages of immobilization. Two types of immobilization methods were used: physical entrapment and adsorption of cells on to inert supports[7]. Agar was used as an immobilization

material for the first method whereas sponge cubes and luffa cyclindrica fibers were used as support materials for the second method.

Agar solution and inoculum were prepared separately. 50 ml of inoculum was prepared. A solution containing 2% agar (130 ml) in a 250 ml Erlenmeyer flask was sterilized and cooled to 40-45 °C. The inoculum was mixed with this agar solution. The mixture was poured into petri dishes, and solidification occured after 10 minutes. Then cubes of $0.8 \times 0.8 \times 0.8$ cm were cut from the solidified agar yeast culture under aseptic conditions. The immobilized cell cubes occupied approximately 60% of the total volume of the fermenter containing 120 ml sterilized growth medium.

In immobilization with sponge cubes, 200 ml of stock culture was used. The culture was then put into five erlenmeyer flasks of 250 ml with equal amounts and sponge cubes of $0.8 \times 0.8 \times 0.8$ cm were added to these flasks. After sterilization, 0.5 g dry baker's yeast was added to each flask. Subsequently, the mixtures were incubated in the rotary shaker for 4 hours in order to provide good immobilization between yeasts and support materials. The sponge cubes containing immobilized yeast were placed in the fermenter under aseptic conditions. Immobilized cell support filled approximately 60% of the total volume of the reactor containing 200 ml of sterilized growth medium.

Immobilization with luffa cylindrica fibers was carried out exactly the same way as with sponge cubes. Luffa cylindrica fibers were cut in cubes of $0.8 \times 0.8 \times 0.8$ cm and used for immobilization.

Ethanol Determination: The amount of ethanol was determined in two different ways: by measuring the weight decrease of the system at certain intervals, and by using the pycnometer method as an analytical method in order to determine the amount of ethanol[6].

According to the following stoichiometric equation, 1 mole of glucose produces 2 moles of CO_2 which in turn escapes from the reactor. This phenomenon is reflected as a weight decrease, which can be correlated to the amount of ethanol produced.

$$C_6H_{12}O_6 \rightarrow 2\,C_2H_5OH + 2\,CO_2$$

In terms of weight, every gram of glucose can theoretically yield 0.51 g of ethanol. It is assumed that 50% of glucose was used to produce ethanol and 50% of it to produce CO_2; thus there is a weight decrease due to the amount of CO_2 removed from the system and the amount of ethanol that was produced.

In the pycnometer method, a clean and dry pycnometer (50 ml) was weighed (W). It was then filled with water up to a level predetermined for each pycnometer. Subsequently each was placed in a water bath at 20°C for 20-30 minutes. At the end of this period, the water above the level of the pycnometer was removed with blotting paper and it weighed (W_{water}). This constant value was proportional to the volume of the pycnometer. For ethanol determination, a 50 ml sample was put into the distillation balloon. Then the pycnometer was rinsed with 25 ml of pure water and was also added to the distillation balloon. After that the balloon was connected to the distillation unit. The sample is distilled until alcohol is obtained up to just under the level of the pycnometer. The pycnometer is filled with distilled water up to level at 20°C. Then it is dried and weighed (W_{sample}). The below equation is used in order to find the density of the sample;

$$d(g/1) = (W_{sample} - W)/(W_{water} - W).$$

From this value the amount of alcohol can be calculated as a volume percent by using the standard tables.

Determination of Efficiency, Yield and Productivity: The efficiency, yield and volumetric productivities of the processes (Table 1) were found by using the following equations:

Efficiency (%) = [(gram ethanol produced)/(gram sucrose used)(0.51)](100)
Yield (%) = (gram ethanol produced/gram sucrose used)(100)
Volumetric Productivity = Ethanol formed (g/1)/Volume of the reactor (1)

Results and Discussion

The operating conditions were determined in 500 ml shake flasks before the fermentations were carried out in the bioreactor. According to the results of these experiments, sucrose was determined as the most effective carbon source and dry baker's yeast was chosen as the microorganism to be used. The growth medium composition was also optimized as above.

The results of all fermentations carried out in the bioreactor are given in Tables 1 and 2. The amounts of alcohol for each immobilized system measured by the pycnometer method are also presented in these tables. Even though higher ethanol yields have been reported by other researchers, this is not relevant with respect to the purpose of this project, since comparison of different operational modes were aimed and optimization was not an issue.

Changes in product concentrations against time using free cells for batch, continuous, fed-batch and semi-continuous processes and immobilized cells for batch processes are shown in Figure 1 and Figure 2.

Table 1. Results of production processes in which microorganisms were used in free form.

	Batch	Continuous	Fed-batch	Semi-continuous
Fermentation time (hr)	96	262	240	237
Reactor volume (1)	0.3	0.3	0.3	0.3
Initial Substrate Concentration (g/1)	220	220	100	220
Added Substrate Concentration (g/1)	-	480	540	586.68
Removed Substrate Concentration (g/1)	-	-	-	146.49
Final Substrate Concentration (g/1)	26.59	164.48	22.71	44.27
Final Ethanol Concentration (g/1)	96.71	267.76	314.06	307.97
Efficiency (%)	86.19	75	96.22	91.46
Yield (%)	43.96	38.25	49.07	46.65
Volumetric Productivity (g/1/hr)	1.01	1.022	1.309	1.299
Amounts of Alcohol (%)	8.76	7.68	9.88	9.37

With free cells, the best results were obtained using the fed-batch process. When the different immobilization materials are compared, it can be seen that both the sponge cubes and luffa cylindrica fibers gave better results than the free cells, whereas agar immobilization proved to decrease yields. The histograms for efficiency (Figure 3 and Figure 4), clearly show that the fed-batch process is the most effective and advantageous. Microorganisms use almost all of the substrate given to the system during fermentation.

This is an expected result since substrate inhibition and catabolite repression were prevented by intermittent feeding of the substrate.

Table 2. Results of production processes in which microorganisms were immobilized.

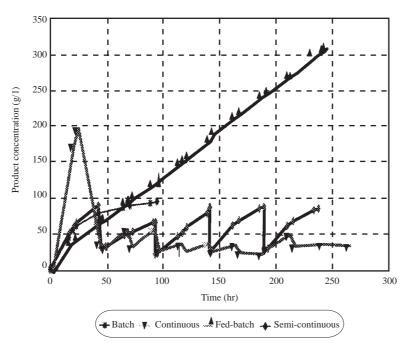|	Agar	Sponge cubes	Luffa Cylindrica Fibers
Immobilization Materials in Batch Processes			
Fermentation time (hr)	96	96	96
Reactor volume (1)	0.3	0.3	0.3
Initial Substrate Concentration (g/1)	220	220	220
Added Substrate Concentration (g/1)	-	-	-
Taken Substrate Concentration (g/1)	-	-	-
Final Substrate Concentration (g/1)	43.54	4.54	19.34
Final Ethanol Concentration (g/1)	88.23	107.73	100.33
Efficiency (%)	78.64	96.02	89.42
Yield (%)	40.11	48.97	45.61
Volumetric Productivity (g/1/hr)	0.919	1.222	1.045
Amounts of Alcohol (%)	8.02	9.85	9.2

Figure 1. Changes in product concentrations against time using free cells for batch, continuous, fed-batch and semi-continuous processes.
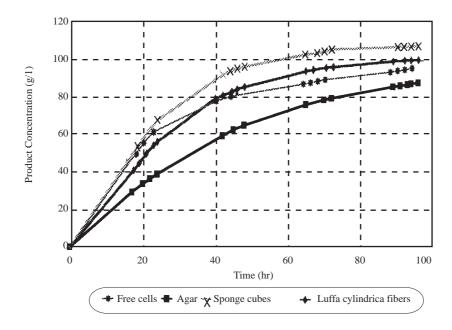
Figure 2. Changes in product concentrations against time using free and immobilized cells for batch processes. Agar, sponge cubes and luffa cylindrica fibers were used as immobilization materials.

Intermittent addition of sucrose improves the efficiency of the fermentation by maintaining a low substrate concentration. Optimization of environmental conditions such as growth and production phase and the age of the culture of the microorganisms is also possible in this mode of operation[10]. However, expensive instruments such as process computers are required to keep the substrate concentration constant and this is the main disadvantage of the process. The other disadvantage of this process is that ethanol concentration becomes inhibitive after a certain level when fermentation time is long. This problem can be solved when a semi-continuous process is used. Both the substrate and product concentration have less inhibitive effects since 2/3 of the reactor was replaced with fresh growth medium every 2 days. Another advantage of this process over batch is that there is no non-productive idle time for cleaning and resterilization. It is also not necessary to control the system as in the continuous or the fed-batch operation. However, higher investment costs are required due to larger reactor volumes. Since long cultivation periods are necessary, there is also a risk of contamination and mutation. When we look at the results of batch fermentations with immobilized cells (Table 2, Figure 4), batch fermentation with *Saccharomyces cerevisiae* immobilized on sponge cubes seems the most effective process. The results of fermentation in which luffa cylindrica fibers were used as an immobilization material (Table 2) are also high when compared to the results with agar. Reasons for this may include loss of activity during immobilization, high intracellular ethanol concentration or diffusion limitations between agar surfaces and microorganisms. However, there was turbidity in the medium during the fermentations in which sponge cubes and luffa cyclindrica fibers were used. This means that microorganisms escaped from the support materials and began to produce ethanol in free form. In an unpublished study in which luffa cylindrica fibers were dyed with methylene blue, these fibers were found to be negatively charged[11]. Since microorganisms from a culture medium are known to be positively charged, a selective adsorption of microorganisms on the support surface is possible. This may explain better results with luffa cylindrica fibers even though there was less turbidity in the culture medium.

From the results of all operations (Table 1 and 2), the fed-batch process seems the most effective and advantageous. The parameters of the semi-continuous process are very close to those of fed-batch

fermentation. From an economic point of view, the semi-continuous process is cheaper than fed-batch, which requires expensive control equipment. However, the semi-continuous process requires equipment which have larger reactor volumes, and this is the main disadvantage of this operation. There are no great differences between the efficiency of batch fermentations with free cells and that with immobilized cells (Tables 1 and 2). If these problems encountered in the immobilization methods can be solved, the advantages of immobilization will result in better operations.
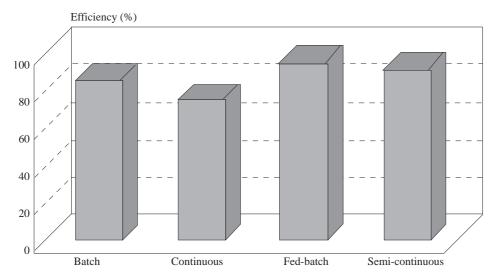

Figure 3. Efficiency histogram for processes in which microorganisms in free form.
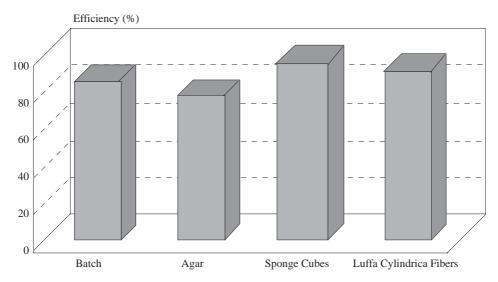

Figure 4. Efficiency histogram for processes in which microorganisms were immobilized on different materials.

The aim of this study was to compare the different modes of operation with respect to efficiency and productivity. The ethanol producion system described was chosen as a means to achieve this end, since *Saccharomyces cerevisiae* is easy to work with. In addition, the fact that the production mechanism is not aerobic enabled us to overlook gas-liquid mass transfer limitations, which generally constitute the predominant reason behind the observed decreases in productivity/efficiency in immobilized systems.

A very thorough literature survey was conducted both for ethanol fermentations in general and with respect to different operational modes, and no comparative study similar to the present one was encountered.

This study aims to fulfill the existing gap and provide some preliminary data for the comparison of different operational modes presently used in industry.

As no similar studies have been carried out previously, the results cannot be verified or discussed in relation to other published data. It is hoped that this study, which provides preliminary insight into the efficiency of various operational modes of bioreactors, will lead to more comprehensive studies using different biological systems.

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