0273-2289/00/84–86/0505/$15.00

Repeated Solid-Phase Fermentation and Extraction for Enzyme Production

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Abstract

Solid-phase fermentation has been found to have a much higher productivity than the popular liquid submerged fermentation in producing cellulase enzymes. The highest reported productivity in the literature for cellulases by *Trichoderma* cultures in submerged fermentation is 158 filter paper units (FPU)/(h·L) of fermenting liquid. From preliminary experiments of solid-phase fermentation in 1000-mL flasks, a productivity of 234 FPU of cellulases/(h·L) of solid-bed volume was obtained. When two novel techniques—pressure pulsation and repeated extraction—were applied, a productivity of 806 FPU/(h·L) was achieved. The same techniques also greatly enhanced the productivity of other enzymes by fungal cultures in solid-phase fermentation.

Index Entries: Solid phase fermentation; pressure pulsation; repeated extraction; fungi; enzyme.

Introduction

Strong efforts were made by American and British scientists and engineers during the World War II to accelerate the production of penicillin for curing battle wounds. The efforts gave birth to the then new technology of cultivating fungal cells submerged in liquid media *(1)*. Ever since, *submerged fermentation* has become the most widely used industrial bioprocessing method. Fungal mycelia grow on the surface of solid substrates more naturally than if they are submerged in liquid. The appearance of molds on moist bread is a common household observation. Rotting of fallen trees in forests provides another simple example. When a piece of wood is totally submerged in water, it is biologically degraded more slowly than a piece of moist wood, not submerged but exposed to the atmosphere. The relatively slow transfer of oxygen from the atmosphere to liquid and then the cells slows down biological degradation when the wood is totally

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submerged. In most submerged fermentation processes, oxygen transfer has been the bottleneck. Mycelia on solid substrate surfaces absorb oxygen directly from the atmosphere without being hindered by surrounding liquid, enhancing cell growth and other metabolic activities. Solid-phase fermentation deserves more attention to achieve its full potential.

Solid-phase fermentation is meant to involve the growth of microbes on moist solid substrates without free-flowing liquid *(2)*. Large-scale solid-phase fermentation does have technical difficulties. First, solid substrates are usually piled together, forming a porous bed that acts as a good heat insulator. For instance, in composting garden wastes, heat is generated by metabolic activities of living cells *(3)*. The heat, not being able to dissipate quickly, causes the temperature of the composting pile to increase, as gardeners have often observed. This increase in temperature helps kill microorganisms and insects. However, if the solid-phase fermentation is intended for high levels of waste digestion or manufacture of certain biological products by pure cultures, the increase in temperature can kill off the biological process prematurely. Second, in solid-phase fermentation, oxygen is supplied to living cells lodged deep in the porous beds by slow diffusion. Moist solid substrates and mycelia often form large aggregates. Cell growth in the aggregate interior is limited by oxygen supply, also reducing the overall process performance.

A common method of improving heat dissipation from and oxygen supply into a composting pile is to turn over the wet solids periodically. Gardeners often do this by hand with simple tools. Manual turnover is not applicable in large-scale operations. More sophisticated systems for solid-phase fermentation are available *(4)*. They involve shallow trays, mechanical turning over, or special cooling devices for enhancing heat dissipation *(4)*. This article describes, instead, a novel, patent pending procedure called *pressure pulsation* that enhances heat and mass transfer in solid piles to improve fermentation performance.

Pressure pulsation involves a simple procedure. Porous packed beds of moist solid substrates in an enclosed cultivation chamber are subjected to periodic pressurization and then depressurization. This is done by placing an on/off valve on the gas exit line of the cultivation chamber, as shown in Fig. 1. When the exit valve is closed, the pressure in the chamber will increase because the inlet gas is flowing into the chamber. When the exit valve is opened, the chamber depressurizes. Convective flows are created when the chamber gas is pressurized and forced into the porous bed and also when the chamber gas is depressurized to release the gas and volatile metabolites from the porous bed. This periodic convective flow into and out of the porous beds enhances heat and mass transport. The periodic pressure pulsation will also help "loosen" and thus prevent the formation of tight cell/substrate aggregates, which also helps gas circulation in the porous bed; this is illustrated in Fig. 2. According to physical theories, heat dissipation by convection is much faster than conduction through porous media. Also, mass transport by convective flow is much faster than molecular diffusion. This has been the basis of a pending patent application.

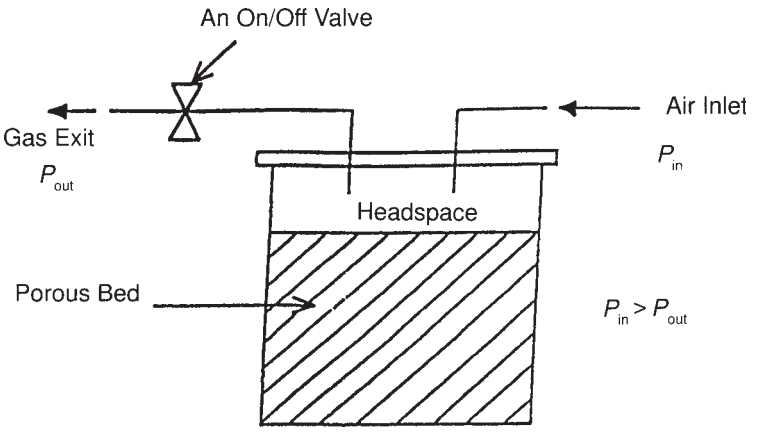

Fig. 1. Simple design of a composting chamber with pressure pulsation in the headspace and the porous bed pulsates between P_{in} and P_{out} by the on/off action of the valve on the gas exit line.

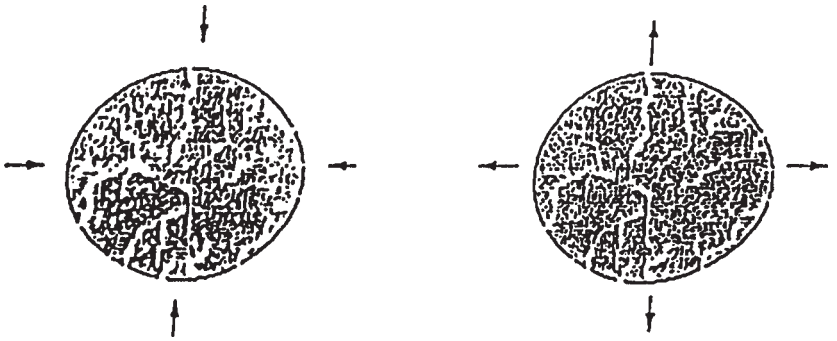

Fig. 2. Diagrams indicating convective flows into a porous aggregate under pressurization and out of the aggregate under depressurization.

As the experimental results given subsequently will show, solid-phase fermentation with pressure pulsation can improve the production of cellulose-hydrolyzing enzymes, cellulases, by 500%. This method requires only simple fermenter construction including an enclosed chamber with an on/off valve attached to the gas exit line. Its initial investment is expected to be much less than for submerged fermentation systems, which require expensive tanks with mechanical agitators as well as power input to drive the agitators.

Renewable materials such as starch from corn wet milling have been used extensively in producing ethanol fuels and value-added products such as lactic acid in the current industry. In the future, when foodstuff needs increase because of the ever-increasing human population, lignocellulosics will become more important as carbon sources to support bioprocessing. The huge amounts of lignocellulosics, including crop resi-

dues and forest rejects as well as the paper and wood wastes in collected municipal solid wastes, are currently largely unutilized. Municipal solid wastes pose serious problems in landfills and other environmental operations. Crop wastes such as straws are often simply burned in the open air to make room for planting the next corp. Concerns about air pollution may soon put an end to this practice.

To make use of lignocellulosics, the overall processing scheme often includes first a hydrolysis step to convert cellulose and hemicellulose into sugars. Fermentation and other bioprocessing methods will then convert the sugars into downstream products such as ethanol and lactic acid. Research in this field in the last 20 yr has resulted in improved performance of the many conversion processes. However, the cost of cellulases is still very high, preventing the development of this entire lignocellulosics-based industry. In a series of workshops *(5)*, experts have identified the high cost of cellulase enzyme to be the most important, single, remaining roadblock to large-scale use of lignocellulosic materials in the production of ethanol fuels. The consensus has been that if the cost of cellulases can be reduced to about one tenth of the current price of $6.00/100,000 filter paper units (FPU), large-scale production of ethanol fuels from waste cellulose materials can begin.

This article reports on the following five recent advances:

1. Use of solid-phase fermentation instead of the traditional liquid submerged fermentation for cellulase production
2. Use of a *Trichoderma* culture that has been found to be a potent producer of endoglucanase and exoglucanase and an *Aspergillus* culture that is a potent producer of cellobiase
3. Pressure pulsation of the gas in enclosed solid-phase fermenters that enhances heat dissipation and oxygen transfer to greatly improve fermentation productivity
4. The technique of cyclic fermentation and extraction to harvest the enzymes to greatly shorten the fermentation time and thus increase the fermenter productivity
5. New applications of the techniques of pressure pulsation and repeated extraction that have been tested in producing other enzymes

Brief Description of Solid-Phase Fermentation

Solid-phase fermentation had been the dominant method of fermentation for many centuries until the advent of submerged fermentation in the 1940s. Solid-phase processes are still used in the production of rice wine, sorghum liquor, soy sauce, cheese, and other traditional fermented food. In the last decade, there has been a resurgence of interest in solid-phase fermentation. It has been investigated for diverse new products including hydrolases, antibiotics, hormones, organic acids, ethanol, and single-cell protein *(4)*. Its many advantages have been recognized. However, commercialization of new solid-phase fermentation arising from these

efforts is entirely absent *(4)*. The final analyses have all been pointing, one way or another, to the difficulty in achieving adequate heat dissipation from large, fermenting, porous solid beds, which prevents scale-up of many promising processes.

Widely different fermentor designs have been used for solid-phase fermentation *(4,6)*. These fermentors can be broadly classified into five types—tray, packed bed, tumble, stirred tubular, and fluidized bed—with widely different methods to enhance heat dissipation. The tray fermentor is probably the oldest type *(6)*. There are also newer versions with modern automation and improved air circulation. Generally, the tray fermentor is labor-intensive. The conveyer fermentor is a modified version of the tray fermentor. For slow solid-phase fermentation processes, the length of the conveyer is very long *(7)*.

The packed-bed fermentor *(8)* is similar in physical appearance to the fermentor with pressure pulsation discussed in this article. The current packed-bed fermentor employs different methods to cool the fermenting solid beds. Forced flow of moist but unsaturated air is a common design to cool by water evaporation. For a deep packed bed, forced airflow will result in channeling and nonuniform distribution when it is operated without pressure pulsation. Another recent design involves expensive flat heat-exchange plates *(4)*. In a 100-L semi–scale up unit, 10 plates are spaced to form 9 compartments in which solid substrates are packed. The plates help remove heat and give good yield of cellulase enzymes. This success illustrates the importance of good temperature control. However, the expensive heat-exchange plates are of limited practical value in large-scale applications.

A tumble fermenter *(9)*, constructed like an ordinary cement mixer, can easily cause injury to the living cells and pack the substrates into tight aggregate balls. The stirred tubular fermenter makes use of the larger external surface and the internal cooling coils to help cooling. Its physical configuration makes solid handling difficult. Finally, the fluidized-bed fermenter *(10)* is not practical in view of the excessive amount of airflow needed to achieve fluidization even though it can ensure a more or less uniform air distribution to solid substrates. Fluidization can also cause serious injury to living cells.

Compared with the five types of solid-phase fermenters we have briefly described, the advantages of pressure pulsation systems become even more evident. The pressure pulsation loosens the packed porous bed, avoiding the formation of aggregates. Its good performance is illustrated by the preliminary experimental results given below.

Brief Description of Cellulases

The cellulose-hydrolyzing cellulases *(11)* consist of three enzymes known as endoglucanase (EC 3.2.14, also commonly known as C_x), exoglucanase (EC 3.2 1.91, also commonly known as C_1), and cellobiase

(EC 3.2.1.21). These enzymes work together to convert cellulose into glucose. Their activities are summarized as follows:

$$\text{Endoglucanase or } C_x: \quad G_n \longrightarrow G_{n-m} + G_m \tag{1}$$

$$\text{Exoglucanase or } C_1: \quad G_m \longrightarrow G_{m-2} + G_2 \tag{2}$$

$$\text{Cellobiase:} \quad G_2 \longrightarrow 2\,G_1 \tag{3}$$

Cellulose is a linear, homopolymer of anhydroglucose represented by G_n in which G represents the formula $C_6H_{10}O_5$ and n stands for the average number of anhydroglucose units per cellulose molecule. The value of n may vary from 2000 to 10,000 in cellulose from different sources. The endoglucanase enzyme cleaves the linear cellulose molecule randomly into shorter chains. The subscripts m can be any integer number with $n > m$. The exoglucanase enzyme cleaves off two units of anhydroglucose from a linear glucose chain to form G_2, which is called cellobiose. Cellobiase then produces G_1, glucose, from cellobiose *(11)*.

Because cellulases are really a mixture, the method for quantifying their activities is highly empirical. The now adopted standard procedure by the National Renewable Energy Laboratory, Golden, CO, for determining the enzyme activities involves hydrolysis of filter paper strips under controlled conditions to produce glucose. Each micromole of glucose produced per minute by a standardized procedure determined by the well-known dinitrosalicylic acid reducing sugar analysis is defined to be one international unit of "filter paper activity" (FPU). This procedure is not ideal because it measures reducing powder but not glucose directly. When an enzyme sample contains very little cellobiase, the hydrolysis will yield mostly cellobiose but not glucose, and thus the results will be misleading. This happens to be the case for the *Trichoderma* culture employed in this study. Additional treatments are needed to correct the results.

Preliminary Results

A *Trichoderma* LORRE C8 culture was selected from a screening program in China for cellulose production. The objective of the screening was for enzyme products to be applied in the softening of textile materials. The selected culture is indeed a potent producer of enzymes containing mostly endoglucanase and exoglucanase activities but barely any cellobiase. Such a cellulase product is ideal for textile application in which the desired outcome is a partial hydrolysis of cotton fibers to soften the material but not to produce large amounts of glucose, as shown by the liquid chromatogram in Fig. 3.

This *Trichoderma* culture has been tested in our laboratory by solid-phase fermentation with substrates consisting of wet ground corncobs mixed with an equal dry weight of wheat bran. After fully grown, the whole culture mixture was air-dried and tested for enzyme activity. This product contained 246 FPU/g and the fermenter (the flasks) productivity could be calculated to be 234 FPU/(h·L) of porous bed volume. As already

Fig. 3. Liquid chromatogram of filter paper hydrolysate by cellulases from *Trichoderma* LORRE C8. Peak at 6.05 is for cellobiose. Peak at 7.45 is for glucose. Other peaks are for unknowns.

mentioned, the liquid-phase submerged process is the most common method of fermentation. This is also true for cellulase production; most of the reports in the literature involved submerged methods. By comparison, the fermenter productivity of the solid-phase fermentation is much higher than the typical productivity of 60–158 FPU/(h·L) in liquid submerged fermentation, reported in the literature and presented in Table 1. The cost of 1 L of stirred tank volume used in submerged fermentation is also more expensive than a 1-L volume in an enclosed box for the solid-phase process. Therefore, even the static solid-phase bioprocess is more productive than submerged fermentation.

An *Aspergillus niger* LORRE 12 culture in our culture collection has been found to be a potent producer for cellobiase when cultivated on the

Table 1
Cellulase Productivity in Liquid Submerged Fermentation with Realistic Substrates

Culture	Substrate	Productivity (FPU/[h·L])	Reference
RUT-30	5% Solkfloc	87	*12*
RUT-30	10% steam-exploded aspen	83	*13*
RUT-30	2.2% steam-exploded poplar	37	*14*
RL-P37	6% lactose	158	*15*
SVG-17	3.7% pretreated grain husk	60	*16*

wet corncob/wheat bran substrate. From these preliminary results, we have been convinced that cultivation on wet solid substrates is a very efficient method of producing hydrolytic enzymes from mycelial types of cells such as *Trichoderma* and *Aspergillus*.

In the small Erlenmeyer flasks, heat dissipation and oxygen transfer are relatively fast. The problem is how to overcome the heat and mass-transfer restriction when the solid-phase fermentation is scaled up. After considering various options, we have developed the idea of "pressure pulsation." This article reports some promising results from tests conducted in solid-phase fermenters of up to 30 L for production of cellulases, cellobiase, and also some other enzymes of industrial interest.

Materials and Methods

Trichoderma culture LORRE C8, from a Chinese screening program, and *A. niger* LORRE 12 culture are kept on slants until ready for enlargement for experimental work. Several moist solid media have been tested for mycelial growth and enzyme production. In one case, ground corncobs and wheat brans (from a local feed store) of equal dry weight were mixed together and water was added at a 3:1 water:solid ratio. The solid substrate at this moisture content does not show free-flowing liquid. It is steam-sterilized in autoclaves at 121°C for 30 min. Corncob is desirable because of its relatively strong rigidity, which helps maintain the physical integrity of the porous solid beds. Only after extensive mycelial growth and extraction of enzymes (to be described later) do the beds start to collapse.

The wet-milling industry processes grain corn to produce high-purity starch. It also generates oil and gluten feed as byproducts *(17,18)*. Corn gluten feed containing about 21% protein has been used as an animal feed. It is a collection of several low-valued intermediate streams in the wet-milling process, mixed and dried together. Among them, new uses for corn fiber has been a topic of much research. Corn fiber contains a low level of protein of 8–10%. As part of the animal feed, it does not contribute much to the overall value of the product. It does contain a substantial amount of carbohydrates, and the corn wet-milling industry has been searching for

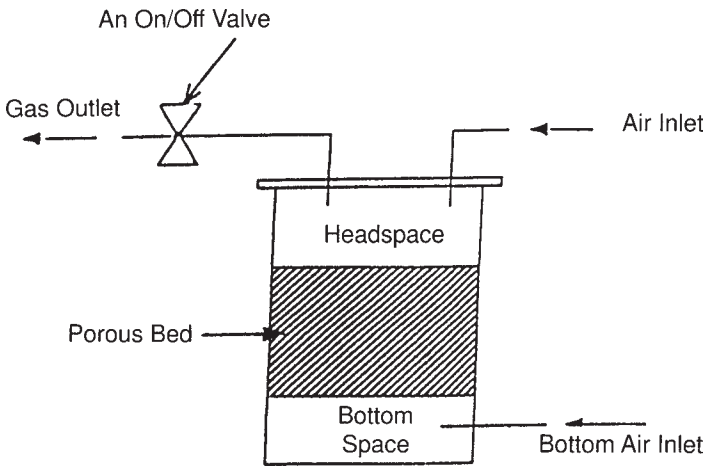

Fig. 4. A 2-L glass jar for solid-phase fermentation equipped for pressure pulsation.

ways to utilize this low-value material. Corn fiber contains the coarse fiber of the hulls of grain corn. This material also provides good bed packing. Corn fiber has also been used as a substrate in solid-phase fermentation. In this case, a typical makeup is as follows: 200 g of corn fiber (14% moisture), 60 g of ground corn grain (local feed store), 30 mL of corn steep liquor (50% solids), 4.0 g of $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4, and 600 mL of water with an overall moisture content of about 75% by weight. The mixture is sterilized at 121°C for 30 min.

Solid-phase fermentation batches were run in vessels similar in construction to that shown in Fig. 1 with a bottom-supported bed. Some runs were conducted in vessels like the one illustrated in Fig. 4, in which a metal screen mesh supports the moist substrate. In this case, it is possible to aerate by forcing air through the bed from the bottom up. A third type of vessel has been constructed in which air can be supplied both over the top of the porous bed and through the packed bed with separate controls. This design allows more flexible control of airflow and thus the bed temperature.

Results and Discussion

Experimental Observations Using a 2-L Glass Jar

A set of experiments were conducted in the type of equipment shown in Fig. 4. In this case, a 2-L New Brunswick glass jar fermenter was refitted for solid-phase fermentation. A metal mesh screen was placed to hold the wet porous solid bed. Air flows into the bottom space and then upward through the porous bed into the headspace. The on/off action of the gas outlet valve created the pressure pulsation of the flowing gas. This arrangement supports excellent cell growth and enzyme production by the *Trichoderma* LORRE C8 culture and the *A. niger* LORRE 12 culture. Without pressure pulsation, the central portion of the bed was tightly packed with

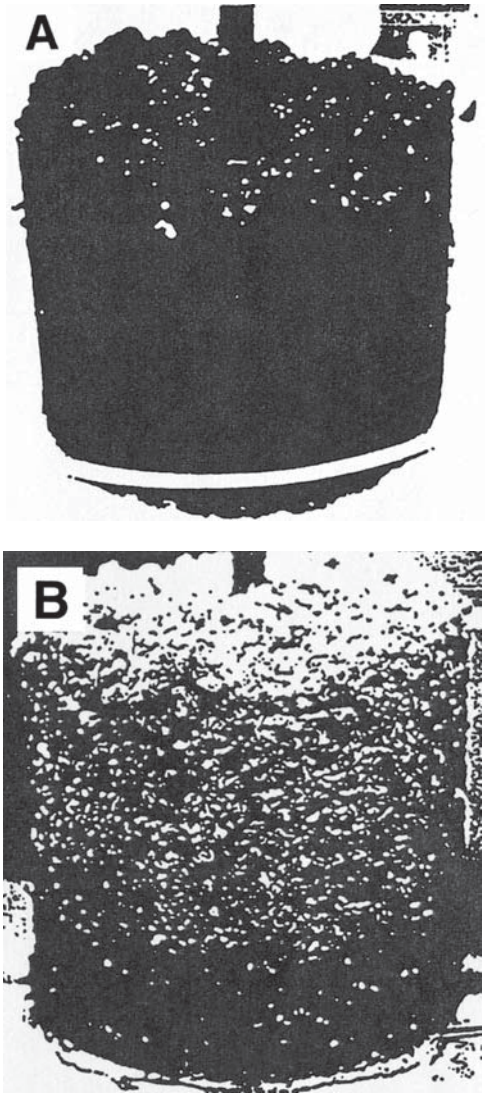

Fig. 5. Comparison of growth of *A. niger* in solid-phase fermentation **(A)** with pressure pulsation resulting in heavy growth with black spores and **(B)** without pressure pulsation resulting in overheating and slow growth.

little growth and no spore formation. Without the pressure cycle, only the portions of the porous bed near the air inlet and next to the glass wall, and the top layer had substantial white mycelial growth, and the bottom portion near the air inlet had black spores, as shown in Fig. 5B. With pressure pulsation, the growth was uniform and heavy throughout. The porous bed turned totally black because of the heavy formation of the black spores by the *A. niger* culture shown in Fig. 5A. With pressure pulsation, the whole bed was "loose," and, in fact, it was difficult to take the bed out of the glass jar without portions of the bed falling off. Similar results can be observed

in *Trichoderma* growth. *A. niger* was chosen to be shown here because the black spores and the white mycelia are more visible.

In this set of experiments, the pressure pulsated between 14.7 and 25 psia with a frequency of 1–3 cpm. Through the wall of the glass jar, we were also able to observe the physical behavior of the porous bed. When the on/off valve on the exit line was opened to depressurize the chamber, the bed always expanded slightly. Repeated pulsation made the bed very loose. On a few occasions, we pulsated the pressure between 14.7 and about 35 psia. In those cases, the expansion was even more visible. However, we did not let it run under high pressure for any length of time because we did not think the glass jar was meant for operation under those conditions. For pressure between 14.7 and 25 psia, the New Brunswick glass jar worked well for extended periods with no problems. For safety reasons, we have locally fabricated, in a machine shop, several new incubation chambers of up to 30 L with steel construction that were tested up to 90 psia.

Repeated Extraction of Enzymes During Fermentation

Once good mycelial growth had been observed, sterile water was pumped into the jar from the bottom up to extract enzymes from the porous bed (*see* Fig. 4). After the liquid extract was pumped out, some liquid was still slowly draining from the bed into the bottom space. When the drainage was complete, airflow was reintroduced to start the bioprocess again. Once good growth alas observed again, the extraction was repeated.

The first extraction was done 3 d after the inoculation. The second extraction was done only 24 h later. This daily harvest of the enzyme extract was repeated several times in both *Trichoderma* and *Aspergillus* experiments. Afterward, the whole residual mass was taken out of the jar and thoroughly extracted with added detergent to make sure that most, if not all, of the surface-adsorbed enzymes were extracted into aqueous solutions. This method of enzyme production was done with the *A. niger* culture for cellobiase and also with the *Trichoderma* culture for the cellulase complex. Table 2 gives the results. From these results and the measured amounts of the solid enzyme products, the enzyme productivity can be calculated to be 806 FPU/(h·L) of porous bed volume for cellulase complex from *Trichoderma*, which is >500% better than the productivity of liquid submerged fermentation reported in Table 1 and 344% higher than that of static run by the same *Trichoderma* culture (in the 1000-mL flasks previously mentioned). In the experiment with the cellobiase-producing *A. niger*, a high productivity of 620 IU (h·L) was achieved. The *A. niger* culture apparently also produced a considerable amount of pectinases and possibly amylases and phytases. However, in this set of experiments, we did not measure the potency of enzymes other than cellobiase, using a reagent grade cellobiose as the assay substrate. One of our early concerns dealt with possible damage of the cells by the repeated pressure pulsation over an extended period. From this set of experiments, we have concluded that the mycelia of the *Trichoderma* and the *Aspergillus* cultures withstood the conditions well without apparent damages.

Table 2
Production of Enzymes with Repeated Extraction

Cellulases by *Trichoderma*		Cellobiase by *A. niger*	
Washing/day	FPU extracted	Washing/day	IU extracted
1st/3rd	14,000	1st/3rd	17,000
2nd/4th	13,000	2nd/4th	17,000
3rd/5th	9800	3rd/5th	17,400
4th/6th	6700	4th/6th	15,200
Final/8th	9800	Final/7th	37,300
Wash with surfactant	16,500		
Drainage	800		
Total	70,600[a]		103,900

[a]Since this *Trichoderma* enzyme mixture contained little cellobiase, the sugars in the hydrolysate are mostly cellobiose with some glucose and cellotriose. After correction as all glucose, the productivity was calculated to be 806 FPU/(h·L) of porous bed volume.

Observation of Bed Temperature

As previously described, we developed the idea of pressure pulsation as a means of controlling the bed temperature and preventing overheating. During the experiments, the bed temperature throughout the runs was measured at two different locations: one at the bed center about 6 cm from the top, and one at the top of the bed. With pressure pulsation, the bed temperature was prevented from rising above 35°C throughout the experiments. Without pressure pulsation, the bed temperature quickly rose above 40–45°C on the second day after inoculation, and occasionally to 60–65°C. Whenever we observed such an increase in temperature, we were unable to obtain good enzyme yields. A pressure pulsation between 14.7 and 25 psia with a frequency of 1–3 cpm was able to control the bed temperature and thus resulted in good enzyme production.

Production of Glucoamylase and Cellobiase by Solid-Phase Fermentation

The *A. niger* LORRE 12 culture is also known to be an amylase producer. In the previously described preliminary experiments, the amylase activities were not measured. A set of more systematic experiments were then carried out with the same culture on the aforementioned corn fiber substrate. The sterilized corn fiber substrate was first inoculated by mixing it with 20 g of pre-prepared, heavily sporulated, solid *A. niger* LORRE 12 culture, and then the mixture was divided into several batches. About 80% of the inoculated solids was placed in the 2-L fermenter shown in Fig. 4 to a bed depth of about 15 cm. The rest of the inoculated solids was divided into four control runs in 250-mL Erlenmeyer flasks as follows:

1. Control no. 1: 30 g with a low bed depth
2. Control no. 2: 60 g with a high bed depth

Table 3
Visual Observation of Solid-Phase Fermentation

	Fermentor		Control no. 1		Control no. 2	
Day	Color	Temp.	Color	Temp.	Color	Temp.
0	Brown	25°C	Brown	25°C	Brown	25°C
1	Light white	35°C	Brown	30°C	Brown	25°C
2	Gray	33°C	Brown	30°C	Brown	25°C
3	Black	33°C	Light white	30°C	Brown	25°C
4	Black	32°C	Light white	30°C	Light white	25°C
5	Black	30°C	White	30°C	White	25°C
6	Black	28°C	White	30°C	White	25°C
7	Black	28°C	Gray	30°C	White	25°C

Table 4
Comparison of Glucoamylase Production

	Fermentor	Control no. 1	Control no. 2	Control no. 3	Control no. 4
IU/g	522.50	213.30	157.40	97.29	153.25
IU/mL	—	—	—	8.27	13.02
IU/mg of protein	2.12	1.52	1.12	0.86	1.15

3. Control no. 3: 40 g plus 100 mL of sterilized water
4. Control no. 4: 40 g plus 100 mL of 5% glucose solution

Controls no. 1, 3, and 4 were incubated on a shaker at 30°C. Control no. 2 was placed next to the 2-L vessel of Fig. 4 as a control of a deep-bed fermentation but with no pressure pulsation applied. The 2-L vessel was aerated with an airflow of 0.5 vvm based on the estimated bed volume. The pressure pulsation was set at about 1.5 cpm between 14.7 and 28.7 psia. After 60 h of fermentation, the color of the porous bed in the 2-L fermenter had become totally dark from spore formation. At that point, 1000 mL of sterile water containing 0.2% $(NH_4)_2SO_4$ was pumped into and out of the fermenter several times to extract the soluble enzymes. After the extraction, the aeration was restarted for additional fermentation. The extraction was repeated at 24-h intervals, and the fermentation was stopped after three washing cycles. The solid residue was then thoroughly extracted to recover all enzymes. All the extracts were then analyzed for enzyme activities. For the solids from the four control runs, 10 g of each wet solid sample was mixed with 100 mL of 0.05 *N* (pH 4.5) citrate buffer in 250-mL Erlenmeyer flasks. The mixture was extracted for 3 h in a 25°C shaker. The solution was harvested after removal of the solids by centrifugation and then analyzed for enzyme activities. In this set of experiments, glucoamylase activities were analyzed by using reagent grade maltose as the substrate, and the same samples were also analyzed for the cellobiase activities by using reagent grade cellobiose as the substrate. Tables 3–6 give the results.

Table 5
Production of Glucoamylase from Solid-Phase Fermentation with Repeated Extraction

Sample	Volume (mL)	IU/mL	Total IU	IU/g (dry)	Percentage	Hour
1st wash	1000	12.29	12,290.0	63.03	12.06	60
2nd wash	1050	10.23	10,741.5	55.08	10.54	84
3rd wash	1060	13.03	13,811.8	70.83	13.56	108
Final solid[a]	14,885.5[b]	4.37	65,049.6	333.59	63.84	132
Total	—	—	101,892.9	522.50	100.00	384

[a]Final solid dry weight: 195 g. Moisture content of final solid culture: (10 – 1.31)/10 × 100% = 86.9%.
[b]Volume = (195/13.1%/10) × 100 mL = 14,885.5 mL.

Table 6
Production of Cellobiase from Solid-Phase Fermentation with Repeated Extraction

Sample	Volume (mL)	IU/mL	Total IU	IU/g (dry)	Percentage	Hour
1st wash	1000	14.07	14,070	72.15	11.61	60
2nd wash	1050	16.40	17,220	88.31	14.21	84
3rd wash	1060	13.20	13,992	71.75	11.54	108
Final solid[a]	14,885.5[b]	5.10	75,916	389.31	62.64	132
Total	—	—	121,198	621.52	100.00	384

[a]Final solid dry weight: 195 g. Moisture content of final solid culture: (10 – 1.31)/10 × 100% = 86.9%.
[b]Volume = (195/13.1%/10) × 100 mL = 14,885.5 mL.

Table 3 provides our visual observation of the progress of cell growth as judged by the color change of the mixture in the batches. The batch in the 2-L fermenter under pressure pulsation turned totally black on the third day. The cultures in controls no. 1 and 2 never turned totally black. It can also be observed that the temperature control by pressure pulsation was also successful in maintaining the temperature at about 30–35°C. As already stated, without pressure pulsation, the bed temperature on the second day would usually rise above 40–45°C. The results in Table 4, on the glucoamylase activities, show that the enzyme production under pressure pulsation was 2.5- to 5-fold that of the controls. The results in Tables 5 and 6 show the glucoamylase and cellobiase activities in the extracts from the 2-L fermenter. The cellobiase activity was about 2.5- to 3.0-fold of that from the static runs reported above.

Economical Consideration

The high cost of cellulases has been identified as the single remaining roadblock to large-scale utilization of lignocellulosic biomass in producing value-added products *(6)*. Preliminary experimental results show that the use of solid-phase fermentation with pressure pulsation and repeated enzyme extraction is capable of increasing the productivity of cellulases greatly. Crude estimates show that the cost-effectiveness of cellulase production can be increased by this solid-phase method by 10-fold (roughly a 5-fold improvement in productivity and a 2-fold improvement in reducing the costs of operation and investment) or more.

From the preliminary experimental data already collected, a low cost of $0.20 per 100,000 FPU of cellulases has been estimated. This involves proper mixing of the enzymes from the *Trichoderma* culture, rich in exoglucanase and endoglucanase, and from the *A. niger* culture, rich in cellobiase. If these results and the cost figures can be verified and repeated with further and more systematic scale-up experimental work, the long-standing bottleneck of the high enzyme cost might be finally overcome. The huge reserve of fermentable sugars held up in lignocellulosic materials will finally become a carbon source for all kinds of energy and petroleum-replacing products. For the 400 million t of annual harvest of farm crops, about 440 million t of crop residues such as cornstalks and wheat straws are coproduced. Assuming 20% will be used to produce ethanol, this will be sufficient to support a huge ethanol industry of an annual capacity of more than 5 billion gal. At a 10% level of blending as an oxygenate additive, this amount of ethanol is sufficient to support the production of 50 billion gal of unleaded gasoline. With the inexpensive cellulases, ethanol can be produced at less than 70 cents/gal.

To illustrate the economics, we use the production of ethanol from sugarcane bagasse as an example. Bagasse is the fibrous residue after sugarcane has been pressed to remove sugar juice. Bagasse is already collected

at sugar mills, and it is currently burned to generate steam for the generation of electricity. We will design a process *(17,18)* involving first taking a part of the bagasse mixed with wheat bran as the substrate to produce cellulases. The enzyme extract and the residual solids containing adsorbed cellulases are mixed with additional bagasse to carry out hydrolysis. Meanwhile, yeast cells are added into the hydrolysis vessel to ferment both glucose and xylose into ethanol in what is called the simultaneous saccharification and fermentation (SSF) process. Recycled carbon dioxide is used as a gas to strip off ethanol from the fermenting broth as ethanol is formed by the yeast. This removes ethanol feedback inhibition on the yeast so that the fermentation step is fast and the overall process is rate limited by the hydrolysis step (which in turn depends on how many units of cellulases are added into the SSF process). Additional bagasse is fed three times into the reactor system until the suspended solids (lignin and other nondigestibles) has accumulated to too high a level, making mixing difficult. The fed batch is then terminated and started over. Ethanol from the system is collected from the carbon dioxide stream and compressed and condensed to a 23% aqueous solution.

Cost Estimate for Cellulases

The amount of substrate in solid-phase fermentation for cellulase production is 700 g/L containing 30% dry materials, which consists of 50:50 bagasse and wheat bran:

$$700 \times 0.3 \times 0.50 = 105 \text{ g of bagasse and also } 105 \text{ g wheat bran/L}$$

The value of bagasse, which is currently use as a fuel, is as follows:

1. 6000 BTU/lb × 2000 lb/t = 12 million BTU/t of bagasse
2. Natural gas at $2.00 per million BTU
3. Estimated value of bagasse as a fuel: 12 million × $2.00 per million BTU = $24/t

Wheat bran can be purchased at $0.05 per pound. The total cost of bagasse and wheat bran per liter of porous bed for enzyme fermentation is calculated as follows:

$$(105/454) \times \$0.012/\text{lb} + (105/454) \times \$0.05/\text{lb} = \$0.0143/\text{L}$$

It takes 2 L of porous beds (one for *Trichoderma* enzymes and one for *A. enzyme*), or what we call 1 L equivalent, to produce a complete enzyme mixture (containing all three components). The total cost for the complete enzyme system is calculated as follows:

$$2 \times \$0.0143 = \$0.0286/\text{L equivalent}$$

The enzyme production per liter equivalent (data from Table 2) for 8 d at 806 FPU/(h·L) is calculated as follows:

$$8 \times 24 \times 806 = \text{about } 150{,}000 \text{ FPU/L equivalent}$$

In the solid-phase fermentation process, other than the substrate, airflow is the major cost. Airflow at 0.1 vvm for 8 × 24 × 60 = 11,520 min to produce 150,000 FPU. Air is compressed to 45 psia to feed the porous bed in the solid-phase fermentor. The work needed to compress the air for 1 L equivalent of fermentor for 8 d is calculated as follows:

$$11{,}520 \times 0.1 \times (2.2/62.4) \times 144 \times 45 \times 2 = \text{about } 5{,}000{,}000 \text{ ft-lb} = \text{about } 3.5 \text{ kW·h}$$

The following equation is used to calculate electricity at $0.04/kW·h:

$$3.5 \times \$0.04 = \$0.14/150{,}000 \text{ FPU}$$

The major cost of substrate and utilities (electricity and others) is estimated to be

$$\$0.03 + (\$0.14 + \$0.04) = \$0.21/150{,}000 \text{ FPU or } \$0.14/100{,}000 \text{ FPU}$$

The crude enzyme extract and the solid residue will be added into SSF system for ethanol production without any intermediate steps of enzyme isolation and purification to save cost. The total substrate and utility cost is assumed to be 70% of the total cost:

$$\text{Total enzyme cost} = \$0.14 \times (100/70) = \$0.20/100{,}000 \text{ FPU}$$

Cost Estimate for Ethanol from Bagasse

The cellulase loading is calculated at 10 FPU/g of total carbohydrates (crude cellulases do contain hemicellulase activities). As already described, the bagasse substrate is added into the SSF system in four shots (initial plus three additions). Bagasse contains on average 80% by weight of carbohydrates (residual sucrose, cellulose, and hemicellulose). The cellulases are all added at the beginning of the fed-batch run. At the beginning of the fed-batch run, the actual enzyme loading in the system is 40 FPU/g of total carbohydrate present in the system. As the old substrate digests, new bagasse is added until the end. Therefore, at any moment of a fed-batch run, the enzyme loading is higher than 10 FPU/g.

1. Cost of bagasse: $24/t
2. Enzyme loading: 2000 × 0.8 × 454 × 10 = 7,264,000 FPU
3. Enzyme cost = (7,264,000/100,000) × $0.20 = $14.53/t of bagasse
4. Total bagasse and enzyme cost = $38.53/t of bagasse

The amount of ethanol to be derived from each ton of bagasse, based on 80% hydrolysis of cellulose, 90% hydrolysis of hemicellulose, and hydrolysate contains 75% xylose. Conversion of xylose into ethanol at 0.4 g/g and glucose into ethanol at 0.45 g/g. Ten percent of total carbohydrate being sugar with a conversion rate into ethanol of 45%.

$$\text{Amount of ethanol} = (2000 \times 0.8 \times 0.5 \times 0.8 \times 0.45) + (2000 \times 0.8 \times 0.4 \times 0.9 \times 0.75 \times 0.4) + (2000 \times 0.8 \times 0.1 \times 0.45) = 532.8 \text{ lb of ethanol or about } 80 \text{ gal}$$

Total bagasse and enzyme cost of ethanol = \$38.53/80 = about \$0.48/gal

The total bagasse and enzyme cost for ethanol is assumed to be 70% of the total production cost:

Ethanol production cost = \$0.48 × (100/70) = 68.5 ¢/gal

Comments on Economical Analyses

The preceding cost estimates were done based on bagasse calculated for its fuel value. In many cases, such as the paper and wood wastes in the collected municipal solid wastes, the substrate does not cost \$24/t. Municipal solid wastes actually cost \$40–\$100 to discharge at a landfill. An ethanol producer using such wastes may get credits instead of paying for the substrates. In that case, the ethanol from this process can be even less expensive. The analyses are admittedly crude, but they give an indication of the large improvement one can get from a low-cost cellulase supply. We suggest on-site production of cellulases that are to be used, without any intermediate steps of enzyme isolation and purification. This is the most inexpensive method of making use of the cellulases.

Among the many products that one can produce from fermentable sugars, ethanol is probably the least economically attractive because it is sold only at about \$1.20 to \$1.60/gal or \$0.18 to \$0.24/lb, and about 50% of the sugar weight is converted into carbon dioxide, which has little market value. Once we have inexpensive fermentable sugars, we can actually produce a number of other higher priced products, made from bioprocesses that are more efficient in weight recovery. For instance, lactic acid is needed for producing polylactide biodegradable polymers with a huge market potential. We can produce lactic acid by a fungal fermentation that can convert glucose and xylose and even arabinose into lactic acid with a theoretical yield of 1.0 g/g (instead of 0.52 g/g for ethanol). In this *Rhizopus* fungal fermentation, 90% of the maximum theoretical yield can routinely be obtained. Lactic acid is currently sold at about \$0.80 per pound. Once large-scale fermentation processes are industrialized, the price might come down to \$0.35 to \$0.40/lb, still much higher than the price of ethanol. If the production of ethanol from lignocellulosics can be made economically competitive, most likely a whole host of other chemicals can be produced and be competitive in the marketplace.

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