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High-productivity fermentation process for cultivating industrial microorganisms

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SUMMARY

High-productivity continuous fermentation processes have been developed for the production of important industrial microorganisms in specially designed fermentors. *Saccharomyces cerevisiae, Pichia pastoris, Kluyveromyces fragilis,* and *Candida utilis* yeasts have been grown in bench-scale fermentors at cell densities of over 120 g/l, while *Escherichia coli, Bacillus megaterium, Methylomonas* sp. and *Pseudomonas putida* bacteria have been cultivated to cell densities of more than 110 g/l. Productivities (g cells per l per h) greater than 25 have been achieved in both bench-scale and 1500-liter fermentors with yeasts, and values as high as 55 have been achieved with bacteria in the bench-scale fermentor. The microorganisms were grown on defined media using ammonia for pH control and as nitrogen source. The fermentor, capable of high oxygen and heat transfer rates, was operated at constant volume with continuous feed and product discharge. The high-productivity process reduces fermentor size, media sterilization requirements, and may under some circumstances eliminate waste and recycle streams. It can also be applied to a variety of biological products.

INTRODUCTION

Fermentation is the key technique in mass-producing microorganisms and microbial products. The batch process widely used to make microbial products is labor-intensive and may sometimes produce products having inconsistent compositions. Continuous culture techniques have not only been used to study the nutritional requirements of microorganisms [1], but also are more efficient means of cultivating microorganisms [6,7]. Although continuous cultivation of microorganisms has been extensively researched, the productivity generally reported by others is low, limited by the low cell densities in the fermentor [6,7]. The mass production of microorganisms and/or their associated products may be carried out either by aerobic or anaerobic fermentation processes. This study deals only with the aerobic process.

The high-cell-density fermentation process not only improves productivity, but also reduces operating cost. For example, in the case where cells are the main products, as with yeasts for flavor en-

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hancement or as a nutrient supplement in foods, a low-cell-density fermentation broth requires further concentration of cells by centrifugation or other means before the products can be economically dried. At high cell densities the concentration step can be eliminated. For some other biological products, such as enzymes, higher cell concentration in the fermentor often translates into higher product concentration that should facilitate the recovery and consequently reduce the manufacturing cost.

Reported here is a study demonstrating greatly improved cell mass productivities with several microbes-substrate combinations using a high-cell-density continuous culture system.

MATERIALS AND METHODS

Yeast and bacterial strains were obtained from several sources, including the U.S.D.A. Northern Regional Research Laboratories (Peoria, IL) and American Type Culture Collection (Rockville, MD). The cultures were deposited in the Phillips Culture Collection (Phillips Research Center, Bartlesville, OK). Working cultures were maintained on agar slants or plates of proper media.

Colonies from a slant or plate were inoculated into a 250 ml Erlenmeyer flask containing 100 ml of minimal medium which contained 0.5–1.0% carbon and energy source and the following ingredients (g/l): KH_2PO_4 5.0; $(NH_3)_2SO_4$ 3.0; $MgSO_4 \cdot 7H_2O$ 0.5; $CaCl_2 \cdot 2H_2O$ 0.1; $FeSO_4 \cdot 7H_2O$ 0.15; $CuSO_4 \cdot 5H_2O$ 0.0125; $ZnSO_4 \cdot 7H_2O$ 0.095; $MnSO_4 \cdot H_2O$ 0.0063; $Na_2MoO_4 \cdot 2H_2O$ 0.0038; and $CoCl_2 \cdot 6H_2O$ 0.0005, as reported previously [8]. The flask was incubated at an optimal temperature in a New Brunswick G-25 shaker. An overnight culture was then used as inoculum for bench-top fermentation.

The bench-top fermentors were custom-built, 4-liter, stirred, baffled fermentors. The mineral media for high-productivity cultivation of the different yeast species have been described previously [6–8]. Initially, the sterile fermentor loaded with 2 liter of medium was inoculated with 100 ml of the overnight culture. The fermentor was operated aerobically at the pH and temperature shown in the Results section until all the carbon source was consumed. Sterile feed was then continuously added at a rate such that the culture was able to completely consume the carbon source. This was normally monitored by dissolved oxygen response. The carbohydrate content of the broth was determined by ion chromatography [5] while the alcohol content was determined by gas chromatography. The ungassed liquid volume of the fermentor was held constant by continuous withdrawal of the fermentor broth. Air was sterilized by filtration and sparged into the fermentor. Gaseous ammonia was used to control the pH and as nitrogen source. The carbon source was always the limiting nutrient throughout the studies.

Mass and heat transfer

Mass transfer was determined by measuring the air flow to the fermentor and the composition of both inlet and outlet air with a Perkin-Elmer gas analyzer. Oxygen transfer rate (OTR) was then calculated: $OTR = f(C_{in} - C_{out})/V$ where f is the flow rate (l/h), C_{in} and C_{out} are the concentration in mmol of oxygen in the inlet and outlet gasses, and V is the ungassed broth volume. OTR was also estimated from yields using the equation $OTR = uX/Yx_{O_2}$ where u is specific growth rate or dilution rate (h^{-1}), X is cell density (g/l) and Yx_{O_2} is yield on oxygen (g cells/mmol O_2). Temperature of the cooling water in and out of the fermentor heat exchanger were recorded and, with the cooling water flow rate, were used to calculate the heat transfer rate and heat load.

Analysis

Samples of the fermentor broth during steady state were withdrawn and dried in an oven at 105°C for at least 12 h to determine the total dry solids in the broth. To determine the cell mass yield, the samples were centrifuged and washed once with deionized water, followed by drying. The yield is expressed as g dried cells/g substrate in the feed.

Table 1

Development of ultra-high cell density for *K. fragilis* grown on whey permeate[a]

Dilution rate (h^{-1})	Whey permeate (g/l)	Lactose (g/l)	Cell density (g/l)	Yield[b] (g/g)	Productivity (g/l/h)
0.33	60	50	23	0.46	7.6
0.33	120	100	46	0.46	15.2
0.28	180	150	66	0.44	18.5
0.24	240	200	90	0.45	21.6
0.20	300	250	112	0.45	22.4
0.17	360	300	132	0.44	22.4

[a] The yeast was grown in bench-top fermentors at 37°C, pH 4.5.
[b] The yield was calculated based on lactose content only.

RESULTS

Development of high-productivity process

A food-grade yeast, *Kluyveromyces fragilis* PC8002, was used in this study. The yeast was grown in continuous culture with reconstituted whey permeate feed containing 6.0% whey permeate solids (60 g dry whey permeate/l). The feed was sterilized in an autoclave by heating at 123°C for 30 min, cooled, and fed to the fermentor at conditions approaching the highest possible dilution rate, 0.33 h^{-1} (Table 1). This rate is determined by the yeast's inherent specific growth rate under these conditions. A cell density of 23 g/l at a productivity of 7.6 g/l/h was obtained. Because 0.33 h^{-1} was the highest possible dilution rate, the only way to improve productivity was to increase cell density. Increasing the whey permeate concentration in the feed stream resulted in increased cell density that was proportional to lactose concentration in the feed. At 30% whey permeate, the cell density reached 112 g/l. Although the cell density could be increased to 132 g/l with 36% whey permeate in the feed, constant heating of the feed reservoir was required to keep lactose soluble. We therefore routinely ran our high-cell-density/high-productivity process at 30% whey permeate.

It should be noted that at each incremental increase in lactose concentration it was necessary to carefully redefine the medium composition so that the mineral nutrient met the requirements of the culture. Normally, the concentration of the mineral salts was increased proportionally with the increase in lactose concentration. Increasing the cell density from 23 g/l to 112 g/l increased productivity from 7.6 to 22.4 g/l/h, a three-fold increase. As the cell density increased, the dilution rate decreased slightly, partly because of the large amount of heat generated. No differences were found in the composition of cells grown at various cell densities.

The process was scaled-up to a 1500-l baffled, stirred tank fermentor containing cooling coils and a mechanical foam breaker. As shown in Table 2, beginning with 6.0% whey permeate in the feed, a cell density of 23 g/l and a productivity of 6.9 g/l/h was obtained at a dilution rate of 0.30 h^{-1}. When the whey permeate concentration was doubled to 12%, the cell density and productivity were also doubled. Further increase in whey permeate concentration in the feed to 24% resulted in a cell density of 90 g/l and a productivity of 21.0 g/l/h. Analyses showed that the oxygen consumption was 1.29 g/g cells and the oxygen transfer rate was 840 mmol O_2/l/h. These results demonstrate that the process developed with the 4-l bench-top fermentors can be scaled-up to a 1500-l fermentor with similar results. We therefore believe that the process developed with the 1500-l fermentor can be further applied to larger fermentors.

Table 2

Cultivation of *K. fragilis* in a 1500-l fermentor[a]

Dilution rate (h^{-1})	Whey permeate (g/l)	Lactose (g/l)	Cell density (g/l)	Yield[b] (g/g)	Productivity (g/l/h)
0.30	60	50	23	0.46	6.9
0.30	120	100	46	0.46	13.8
0.23	240	200	90	0.45	21.0

[a] Growth conditions: 37°C, pH 4.5.

[b] The yield was calculated based on lactose content only.

Oxygen transfer rate

Oxygen is critical to aerobic fermentation processes. A fermentor to be used to grow yeast cells to high cell density/high productivity must have exceptionally good oxygen transfer. A combination of high-level agitation, a good air sparging system, and pressure are required to meet the oxygen demand of the microbial cells. The results shown in Fig. 1 were obtained with *K. fragilis* grown in the 1500-l fermentor. The OTR at a low productivity of 7.0 g/l/h was 240 mmol O_2/l/h, which increased to 840 mmol O_2/l/h as productivity increased to 21.0 g/l/h. These results demonstrate that our fermentation system has the exceptional oxygen transfer required for high-productivity/high-cell-density fermentations.

High productivity cultivation of other yeasts

A strain of torula yeast, *Candida utilis* PC8008, was grown to high cell density in bench-top fermentors in continuous runs lasting as long as 60 days, using sucrose as sole carbon and energy source. After successful bench-top experiments, the fermentation was scaled up to the 1500-l fermentor. The results shown in Table 3 were obtained at a dilution rate of 0.19 h^{-1}, pH 4.0 and 32°C. The dissolved oxygen level was maintained at 20–25% of saturation. As the results show, the fermentor broth contained 138.4 g/l dry washed cells, which corresponds to a yield of 0.5 g/g sucrose and a productivity of 26.4 g/l/h. An OTR of 692.7 mmol O_2 was delivered by the fermentor, and the system removed 72.7 kcal/l/h of heat. The OTR and productivities obtained with torula in the 1500-l fermentor are similar to those illustrated in Fig. 1. Since this fermentation process does not require preconcentration of the product before it is dried, the actual product recovered is the total dry solids contained in the fermentor broth, which was 150 g/l. This represents a productivity of 28.5 g/l/h. At a cell density of 40 g/l, even at the highest dilution rate of 0.3 h^{-1}, the productivity would be only 12 g/l/h, less than half that of the high-cell-density process.

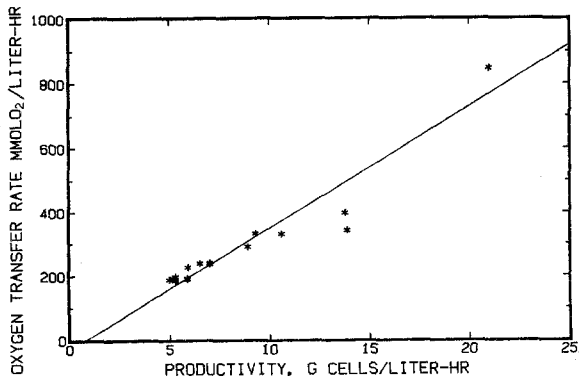

Fig. 1. Oxygen transfer rate vs. productivity. Yeast (*K. fragilis*) was grown in the 1500-l fermentor as described in the text. The productivity was determined as in Materials and Methods.

Another yeast strain, *Pichia pastoris* PC4002, a methanol-assimulating yeast, was also grown in the 1500-l fermentor at a dilution rate of 0.11 h^{-1}, pH 3.5 and 30°C. Cell densities as high as 126 g/l have been obtained. As shown in Table 3, at a cell density of 105 g/l, the productivity was 11.6 g/l/h, which is almost three-times the 4.2 g/l/h obtained at a cell density of 32 g/l with the highest possible dilution rate of 0.13 h^{-1} (data not shown). The fermentor system was capable of delivering an OTR

Table 3

High-productivity process for growing yeast

	C. utilis PC8008[a]	*P. pastoris* PC4002[a]	*S. cerevisiae* PC6005[b]
Substrate	Sucrose	Methanol	Sucrose
Substrate conc. (g/l)	275	263	275
Dilution rate (h^{-1})	0.19	0.11	0.20
pH	4.0	3.5	4.0
Temperature (°C)	32.0	30.0	32.0
Cell mass (g/l)	138.8	105.0	138.0
Productivity (g/l/h)	26.4	11.6	27.6
Yield (g/g)[c]	0.5	0.4	0.5
OTR (mmol O_2/l/h)	692.7	880.0	950.0
Oxygen consumption (g O_2/g cells)	0.82	2.42	1.10
Heat release			
kcal/l/h	72.7	109.0	110.0
kcal/mol O_2	110.1	123.9	115.8

[a] Data obtained with the 1500-l fermentor described in text.
[b] Data obtained from bench-top fermentor.
[b] Based on substrate concentration in the feed.

of 880 mmol O_2/l/h while removing 109 kcal/l/h of heat produced from the fermentation.

Also shown in Table 3 is the cultivation of *Saccharomyces cerevisiae*, the most extensively studied and used industrial yeast species, employing the high-productivity system. A pure culture of *S. cerevisiae* was isolated from locally purchased Baker's yeast, and carried the Phillips Culture Collection number PC6005. The fermentation was carried out at a dilution rate of 0.2 h^{-1}, pH 4.0 and 32°C. The cell density reached 138 g/l, having a productivity of 27.9 g/l/h. For a commercial production of *S. cerevisiae*, this high-productivity process would reduce concentration steps and the quantity of waste material generated.

The yield factor was very important in the study. Higher yield means not only more cells per unit substrate used but also lower oxygen requirement during cultivation. Table 3 also shows that with 0.5 g/g yield for *C. utilis* and *S. cerevisiae*, the oxygen consumption was 0.82 and 1.10 g O_2/g cells produced, while *P. pastoris*, having a yield of 0.4 g/g, required 2.42 g O_2/g cells produced. Additionally, lower oxygen requirement translates to less heat release during growth. Production of *C. utilis*, for example, although having a productivity more than twice that of *P. pastoris*, produces only two thirds of that produced by *P. pastoris*. Lower heat generation reduces cooling costs.

High productivity process for growing bacteria

The next concern regarding this high-productivity process was whether it could be applied to growing bacteria. Yeasts are hardy microorganisms and tolerate vigorous agitation better, and are probably more resistant to by-products produced during growth than bacteria. Results from bench-top fermentation for growing some bacteria are shown in Table 4. First, *Escherichia coli* K12 ATCC10798 was grown on glucose at a dilution rate of 0.37 h^{-1}, pH 6.5 and 38°C to a cell density of 110.5 g/l at a productivity of 40.9 g/l/h. The same strain was also grown to a productivity of 55.4 g/l/h, but the yield decreased from 0.44 g/g to 0.39 g/g. However, the medium used for this test was not optimized for *E. coli* at the test conditions.

The process was then applied to the growth of *Bacillus megaterium* QMB1551 at 0.15 h^{-1} dilution rate, pH 6.5 and 38°C with the same medium, except sucrose was substituted for glucose. As demon-

Table 4

High-productivity fermentation process for cultivating bacteria[a]

	E. coli K12 ATCC10798		*B. megaterium* QMB1551	*Methylomonas* sp. NRRL15416	*P. putida* PPS597
Carbon source	Glucose	Glucose	Sucrose	Methanol	Sucrose
Carbon source conc. (g/l)	250	250	250	233	270
Dilution rate (h^{-1})	0.37	0.56	0.15	0.25	0.14
pH	6.5	6.5	6.5	6.5	6.8
Temperature (°C)	38.0	38.0	34.0	30.0	32.0
Cell mass (g/l)	110.5	98.9	110.0	114.0	83.6
Productivity (g/l/h)	40.9	55.4	16.5	28.4	11.7
Yield (g/g)[b]	0.44	0.39	0.44	0.49	0.31

[a] The bacteria were grown in bench-top fermentors as described in Materials and Methods.
[b] Based on substrate concentration in the feed.

strated in Table 4, the cell density was 110 g/l with a 16.5 g/l/h productivity. This strain produces extracellular material that increases the fermentation broth viscosity, thereby reducing mass transfer. With other strains of bacilli that do not produce viscous extracellular products, productivity may be doubled or even tripled.

Because methanol was once thought to be a cheap feedstock for producing microbial products, such as single cell protein [2], we did extensive research on screening methanol-utilizing bacteria and developing a fermentation process for growing these bacteria. Some of the results obtained with a strain of *Methylomonas* sp. are shown in Table 4. At 0.25 h^{-1} dilution rate, the bacterium was grown to a density of 114 g/l, which represents a productivity of 28.4 g/l/h. The production of this bacterium was successfully scaled-up in the 1500-l fermentor.

Finally, the medium for growing *Methylomonas* sp. was used to grow an auxotroph strain of *Pseudomonas putida(his)*, employing the high.-cell-productivity process except that sucrose was the carbon source. The strain that we worked on produced a large quantity of extracellular materials because the spent medium fraction (supernatant fraction after centrifugation) contained 74.7 g/l solids. This large amount of soluble products exuded from the cells explains the low cell yield of 0.31 g/g substrate. However, the results demonstrate that the fermentation process is suitable to improve not only the productivity of bacterial cells, but also that of soluble products. Again, the dilution rate could have been increased, if the medium was optimized for *P. putida*.

DISCUSSION

In a continuous culture, the productivity, P, is defined as $P = DX$, where D is dilution rate and X is cell density. Increases in D and/or X will result in higher productivity. Since D also equals the specific growth rate, u, of the microorganism, the dilution rate is limited by the microorganism's intrinsic characteristics. Once u_{max} or D_{max}, maximum dilution rate, is achieved, P can only be increased by increasing cell density, X.

Assuming continuous culture at steady-state conditions with carbon source as the limiting nutrient and negligible maintenance requirement, cell density is the product of cell mass yield and initial carbon source concentration. If yield remains constant, cell density can be increased by increasing the carbon source concentration in the feed. To accommodate the increased substrate concentration, the concentration of mineral nutrients also needs to be increased. We found that some subtle adjustments of mineral concentration were very important in achieving high cell densities, and thus high productivities.

Microorganisms that produce waste products that are toxic or inhibitory to their growth generally can not be grown to high cell density [4]. Although they grow well at low cell densities, they fail to grow well as the cell density increases due to the increase in waste product concentration.

Heat transfer is one of the most important factors in the fermentation process. Increasing cell density results in increased viscosity of the fermentation broth and decreased heat transfer. Heat released during the fermentation must be efficiently removed to ensure the proper growth and yield. Data presented show that our fermentation system is capable of providing both the oxygen and heat transfer required for a high-cell-density, high-productivity fermentation process. The oxygen consumption, g O_2/g cells, in our process is very comparable to those reported in the literature [9].

The cell density in the fermentor could be increased by other means, such as using oxygen-enriched air to improve the oxygen transfer, and recycling the yeast cells or medium. For instance, Lane [3] reported that up to 90 g/l of yeast cell mass could be obtained by dialysis. However, this involves the recycle of medium through membrane, and additional equipment is required. It also generates a large waste stream, as does centrifugation. Furthermore, he reported a marked and progressive decrease in cell mass yield as the cell density was increased. With our process, there is no need to recycle the medium and, as shown in the Results section, there is little reduction in cell mass yield as the cell density is increased to over 130 g/l.

Collectively, the results reported demonstrate that the productivity of a variety of industrial microorganisms, including yeasts and bacteria, can be improved by the high-cell-density, high-productivity process. This process has substantially less fermentation-liquor effluent volume and make-up water for medium formulation. This would mean savings in reduced sizes of sterilization, fermentation, separation, and purification equipment that are very important in producing biological products.

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