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Stabile Fermentation of Citric Acid Using Immobilized *Saccharomycopsis lipolytica*

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The effects of media composition on citric acid fermentation using surface immobilized *Saccharomycopsis lipolytica* were studied. The use of the standard medium for these organisms resulted in rapid decrease of citric acid production and a transformation of immobilized cell morphologies from a yeast-type to a mycelium-type. When the standard medium was enriched with vitamins, trace minerals, a growth factor and ammonium to form a Vigorous Stationary Phase (VSP) fermentation type medium, relatively stable citric acid production (10 mg/l·h) was obtained. Using the VSP type medium, the surface immobilized cells also retained their yeast-type form.

In spite of the potential advantages of immobilized viable cell reactors, such reactors will not become commercially prevalent until a number of operational problems are solved. The most significant of these problems is the lack of long-term stability. Instability of long-term operations is usually the result of cellular growth. This growth can cause plugging/channeling in the reactor, the release of cells into the effluent, and in the case of a gel matrix, disruption of the gel. An immobilized non-grwoing viable cell reactor can avoid such problems. However, many researchers report difficulties in maintaining cellular activity under non-growth conditions (3, 5, 11).

A technique for maintaining cellular activity by the periodic addition of nutrients to entrapped viable cells was suggested by Forberg *et al.* (4). This method could reduce the problems, such as overgrowth of cells at the surface of the matrix and starvation of cells inside the matrix, which would occur if nutrients were continuously fed to the entrapped cells.

Recently Kim *et al.* determined the nutritional and environmental requirements of *Saccharomycopsis lipolytica* to maintain active fermentation for long periods of time without undergoing significant growth or death (7, 8). This type of fermentation has been named Vigorous Stationary Phase (VSP) fermentation. VSP fermentation was developed using a Continuous-Stirred-Tank-Membrane-Reactor (CSTMR) system in which the *S. lipolytica* were in free suspension. Although the VSP fermentation requirements were developed using free suspension cells, the data should also be applicable to immobilized cells if potential problems with mass transfer effects are resolved. In immobilized cell reactors (ICR), the environment for individual cells can be substantially different from conditions in the bulk fermentation broth due to resistance to mass transfer.

A VSP fermentation can only be accomplished when the ICR and media are designed such that the nutritional and environmental requirements of individual immobilized cells are met. The VSP fermenntation medium developed using the CSTMR could be used without modification in an ICR designed with negligible resitance to mass transfer. Internal mass transfer resistances can be eliminated if the cells are surface immobilized in a monolayer on a nonprous solid such as a glass bead. External mass transfer limitations can be minimized if the stagnant film surrounding the monolayer is negligible, which can be accomplished by having a relatively high flowrate of medium past the monolayer of cells.

In this paper, the effects of medium composition on the stability of an immobilized cell reactor are reported. The cells were immobilized in a monolayer on solid glass beads and packed into a tabular reactor. Medium was recirculated at a high flowrate and was periodically

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replaced. This immobilized cell reactor is the equivalent of what is commonly called a differential reactor (7).

MATERIALS AND METHODS

Microorganism

A culture of *Saccharomycopsis lipolytica* D. 1805 (ATCC 20390) was obtained from the American Typee Culture Collection. Long-term stock cultures were preserved at 4℃ on YM-broth agar slants, which were subcultured at intervals of one to two months.

Medium Preparation

Three types of medium were used in the immobilized cell reactor experiments: the standard medium, an enriched medium and a VSP medium. In addition, a seed culture medium was used to grow the seed culture.

The standard medium was specified by Briaffaud and Engasser for *S. lipolytica* growth and subsequent fermentation of citric acid (6). Growth ceased and citric acid production was reported to begin after ammonium was totally depleted. The composition of the standard media for citric acid production (deficient in ammonium) is shown in Table 1. To make the enriched media, vitamins, a growth factor (mesoinositol), and trace minerals (NaCl, $CaCl_2$, $ZnSO_4$, and $CuSO_4$) were added to the standard media. To make VSP medium, ammonium nitrate was added to the enriched medium.

Table 1. Composition of seed culture and reactor feed media

Component	Media			
	Seed (g/l)	Standard[1] (g/l)	Enriched (g/l)	VSP (g/l)
KH_2PO_4	2.0	2.0	2.0	2.0
H_4NO_3	1.0	0.0	0.0	*
NaCl	0.01	0.0	0.1	0.1
$CaCl_2$	0.06	0.0	0.06	0.06
$MgSO_4$	2.0	2.0	2.0	2.0
$FeSO_4$	0.01	0.01	0.01	0.01
$MnSO_4$	0.01	0.01	0.01	0.01
$ZnSO_4$	0.002	0.0	0.002	0.002
$CuSO_4$	0.002	0.0	0.002	0.002
Thiamin-HCl	0.01	0.01	0.01	0.01
m-inositol	0.125	0.0	0.125	0.125
pyridoxin-HCl	0.625	0.0	0.625	0.625
nicotinic acid	0.5	0.0	0.5	0.5
Ca-D-pantotenate	0.625	0.0	0.625	0.625
D-biotin	0.0125	0.0	0.0125	0.0125
Glucose	30.0	20.0	20.0	20.0

[1]Media as specified by Briaffaud and Engaser (6).

*Vaires depending on experimental

Medium components were sterilized at 121℃ for 20 minutes excluding $FeSO_4$ and vitamin solutions. These were filtered through a 0.2 μm membrane and added to the previously autoclaved medium. Temperature and pH were maintained at 30℃ and 4.9, respectively.

Preparation of the Reactor

Solid galss beads (0.725 mm in diameter) were purchased from the Delong Equipment Co. (Atlanta, Georgia). A 25% gelatin solution was first autoclaved and then cooled down to 28℃ in a water bath. Then the sterilized glass beads were added to the gelatin solution and the mixture was homogenized.

When the gelatin bead mixture began to solidify (10~15 minutes after mixing), it was mixed with cold, sterilized, distilled water (3~4℃). After 10 minultes of gentle stirring, the gelatin coated glass beads were packed into the glass column (40 cm×2.5 cm diameter). These packings were dried under vacuum for 24 hours.

After drying, the gelatin-coated beads were suspended in a 3% glutaraldehyde solution for 4 hours. Then the column was washed with a 0.05 M phosphate buffer solution (pH 6.5) until free glutaraldehyde was not detected by 2,4-dinitrophenylhydrazine. Again the column was dried for 24 hours. Finally the reactor assembly (see Fig. 1), including the column reactor and the reservoir, was sterilized using ethylene-oxide gas for 15 minutes. Aseptic techniques were used during all steps of the reactor preparation.

Immobilization Procedure

S. lipolytica from a slant culture was inoculated into 30 ml of YM broth. Ten ml of this inoculum was transferred into 100 ml of seed culture medium and grown at 30℃ for two days. 100 ml of the seed culture was then inoculated into a 2 liter New Brunswick fermenter (Edison, New Jersey, U.S.A) with 1 liter working volume. During the culture, pH was maintained at 4.0 by the addition of NaOH. Dissolved oxygen in the medium was monitored and maintained at essentially saturation levels. After two days of cultivation, cells were harvested and resuspended in the 0.05 M phosphate buffer (pH 6.5) to obtain a cell concentraction of 10^9 cell/ml. Cell suspensions were pumped into the packed column and remained for 4 hours under quiescent conditions to facilitate cell attachment. After 4 hours, unattached cells were removed by washing with the standard medium.

Reactor Operation

The vessels for the experiments were water-jacketed and 30℃ water was circulated through the jackest to maintain a constant fermentation broth temperature (see Fig. 1). Humidified air was supplied to the sparger in the recirculating reservoir. The total volume of the reaction medium was 155 ml. Medium was recirculated by a peristalic pump to reduce the mass transfer limitations

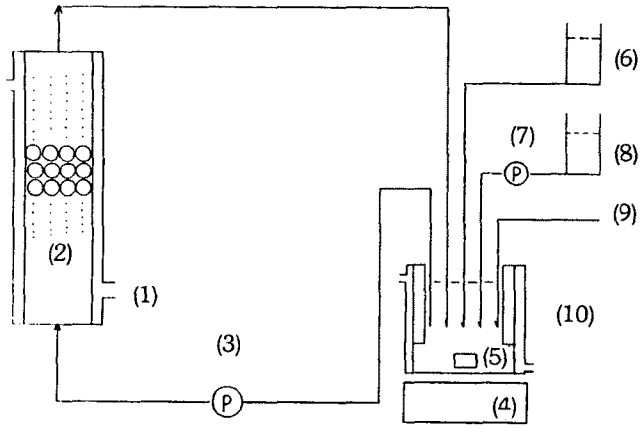

Fig. 1. Schematic diagram of immobilized recirculation reactor.
(1) Water jacket (2) Packed column (3) Recirculation pump (4) Stirrer (5) Magnetic bar (6) Growth media (7) Peristaltic pump (8) Ammonium (9) Air (10) Baffle.

and to provide oxygen. The recirculation rate was 43 ml/min. In the case of the YCB medium, NH_4NO_3 was fed continuously to the reactor at a rate of 56 μg/(10^{10} cells·h). The amount of ammonium nitrate required by the cells was determined in previous investigation (5, 6). The reactor was operated in a continuous semi-batch mode. At five day intervals the medium was withdrawn from the reactor and replaced with fresh medium. A sample for analysis was taken daily.

Analytical Methods

Glucose and citric acid concentrations were determined by colorimetric methods as described by Kim *et al.* (8). By-products were found by HPLC analysis as described by Kim *et al.* (7). Dissolved oxygen was measured by a polarographic oxygen probe (Edison, New Jersey, U.S.A.). Determination of viable cell concentration were determined as described by Doran *et al.* (2).

Electron Microscopy

Glass beads covered with *S. lipolytica* cells were prepared for scanning electron microscopy. Glass beads were fixed 3% glutaraldehyde solution overnight and washed using a serial dilution of ethanol (50, 70, 90, 100%). After washing with ethanol, samples were dehydrated under vacuum for 4 hours at room temperature. The samples were then mounted and coated with a thin layer of gold. The samples were examined with a CWIK-SCAN scanning electronic mkicroscope (Coaltes and Welters, Sunnyvale, California) with a 16 KV acceleration voltage.

RESULTS AND DISCUSSION

Reduction of Resistance to Mass Transfer

The immobilized cell reactor was designed to have negligible internal and external resistances to mass transfer. No internal resistance to mass transfer existed since a monolayer of cells was attached to the surface of solid glass beads. The external resistance to mass transfer was minimized by recirculating the fermentation broth at a high flowrate. Under the chosen conditions, the effectiveness factors for essential nutrients, glucose and oxygen, were calculated to be approximately unity, thus indicating that the external mass transfer resistance would be negligible.

To confirm that the mass transfer limitations were negligible, the flow rate of recirculation was increased three times and, in separate experiments, the oxygen tension in the bulk fermentation broth was increased three fold. Essentially constant reactor productivity was observed. The oxygen tension of the media did not significantly change from the inlet to the outlet of the packed column. These results strongly indicated that the external mass transfer resistances were negligible.

Activity of Immobilized Cells

In order to assess the effect of the immobilization procedure on cellular activity, unattached cells were obtained by washing the reactor after four hours of the binding reaction. The glucose consumption rate (GCR) and citrate production rate (CPR) of the unattached cells were compared with those of a typical fermentation (Table 2). Both the GCR and CPR were reduced during the immobilization procedure. Before immobilization, the cells were harvested from an aerobic fermenter and during the immobilization procedure, they were under essentially anaerobic conditions for four hours. During this anaerobic period, cellular metabolism was apparently changed and thus the citric acid productivity was reduced.

An analysis of the fermentation broth confirmed a change in cellular metabolism. After the immobilization procedure, the free and immobilized *S. lipolytica* cells produced pyruvic acid in addition to citric acid. The period of anaerobic conditions probably reduced the citrate synthase, the pyruvate dehydrogenase, or another enz-

Table 2. Reduction of cellular activity during preparation of ICR

	GCR	CPR	Yield
	(μg/10^8 cell·h)		(g acid/g glucose)
Before anaerobic period	95	42	0.44
After anaerobic period	74	7.2	0.097

Table 3. Rates of free and immobilized cells

	GCR ($\mu g/10^8$ cell·h)	CPR	Yield (g acid/g glucose)
Free	74	7.2	0.097
Immobilized	52	2.4	0.046

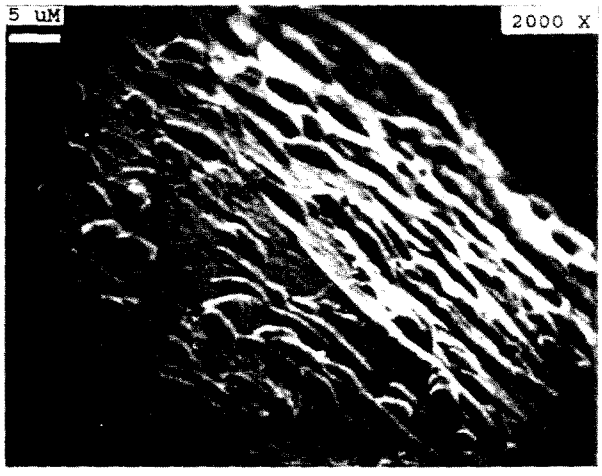

Fig. 2. Attachment of *S. lipolytica* on the glass bead.

yme(s) related to the oxidative phoshorylation significantly more than the glycolysis-related enzymes.

The critical effects of oxygen on citric acid production by *A. niger* was investigated by Kubiek *et al.* (9). If *A. niger* are deprived of oxygen for a few minutes, citric acid production is retarded and does not recover completely even after the resumption of aeration. However glycolysis is not inhibited significantly. In *S. lipolytica*, a strict aerobic yeast, the cessation of the oxygen supply for four hours during the immobilization procedure probably reduced the citric acid productivity.

After determining the effects of the immobilization procedure, GCR and CPR were determined for the immobilized cells and unattached cells. The cell unmber in the ICR was found by disassembling the ICR after 40 hours of operation. The GCR for the immobilized cells was 71 percent that of the unattached cells (Table 3). Also a decrease of yield was observed. Since the reactor was designed to minimize the mass transfer effects, the reduction in GCR appears to be due to the attachment of cells on the solid surface. Scanning electron microscopy of immobilized beads showed that the cells were closely attached to the surface of the matrix resulting in the reduction of available cellular surface area for nutrient uptake. This reduction of the available cellular surface probably caused the reduction of the glucose uptake rate (see Fig. 2).

Effects of Media on Cell Morphology

Cells in the late log phase were harvested and attached to glass beads and medium was continuously supplied in a semi-batch manner. Since the cells were transferred from the batch reactor shortly after the medium nitrogen was depleted, cellular growth was observed for a short period of time. This growth appears to be the result of excess intracellular nitrogen being converted into additional cells. Similar cell growth was also observed in suspension culture (6, 8).

The medium composition was found to affect the cellular morphology. When the standard medium was used, scanning electron microscopy revealed the cells formed netlike structures by mycelial development along the surface of the glass beads. However, when either the enriched medium or the VSP medium was used, the cells retained their yeast-form (see Fig. 3).

Mycelial-type growth of *S. lipolytica* has been previously reported when the medium was enriched with an olive oil and casamino acids, factors which increased the extracellular lipase production by the yeast. Also the absence of cysteine was found to cause the development of *S. lipolytica* mycelia (11). However the mechanism of mycelial development appears to be a very complex response to environmental factors. Additional research is required to better understand the mechanisms of mycelial development.

Effects of Media on the Stability of GCR and CPR

Three different types of medium were used to investigate the effects of medium composition on the stability of citrate production by immobilized *S. lipolytica*: the standard medium, an enriched medium and a VSP medium. In Fig. 4 and 5, the stability of glucose consumption rates and citrate production rates are shown using the different media.

When only the standard medium specified by Briaffaud and Engasser was continuoulsy fed into the reactor, citric acid production stopped after 10 days of operation. During this period, the glucose consumption rate also decreased. *S. lipolytica* in continuous suspension culture exhibited similar behavior using the standard medium (8). The reason for this might be that the standard medium, orignally developed for batch culture, not for continuos culture, is relatively simple and deficient in critically required compounds.

When the standard medium was enriched with vitamins, a growth factor and minerals, the stability of citrate production was improved to some extent. However after 13 days of production, GCR and CPR rapidly decreased. When the enriched medium was supplemented with NH_4NO_3 to form VSP media and this medium was fed into

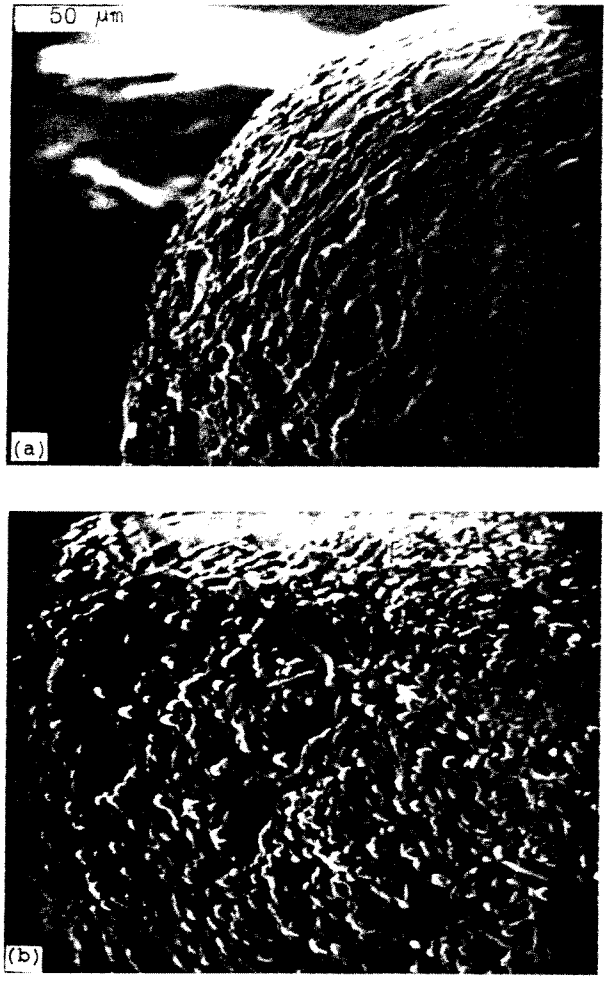

Fig. 3. Effects of media composition on cell morphology.
(a) Mycelial development in simple media, (b) Development of yeast-form cells in enriched media (500×).

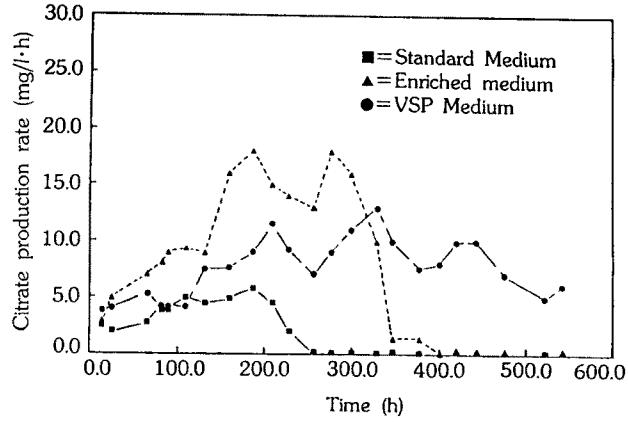

Fig. 4. Effects of media composition on the glucose consumption rate in an ICR.

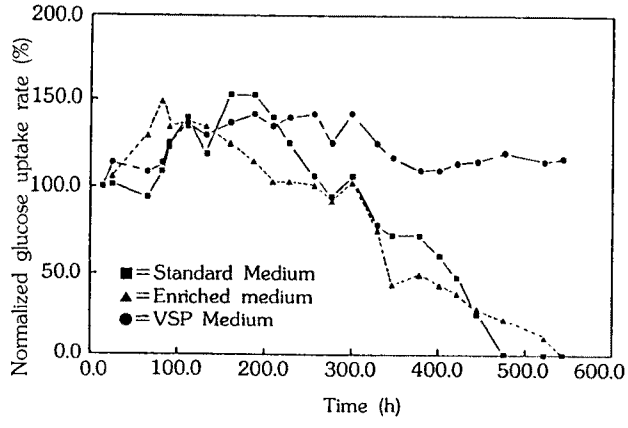

Fig. 5. Effects of media composition on the citrate production rate in an ICR.

the reactor continuously, relatively stable GCR and CPR for over 23 days were obtained. This finding is similar to the results obtained for suspension cultures using the CSTMR (8). The initial increase in the GCR and CPR might be due to cellular grwoth from the intracellular nitrogen.

During the continuous supply of ammonium, negligible suspended cells were observed in the effluent. Also the number of immobilized cells per unit volume of the reactor remained essentially constant with the ammonium addition. From these results, the addition of ammonium to the nitrogen-limited *S. lipolytica* was apparently sufficient for the cells to maintain cellular activity without significant cellular growth. With a stable cell population and cellular activity, the immobilized cell reactor is the equivalent a heterogeneous catalytic reactor.

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