

Direct Fermentation of Potato Starch to Ethanol by Cocultures of *Aspergillus niger* and *Saccharomyces cerevisiae*†

MOHAMED M. ABOUZIED AND C. ADINARAYANA REDDY*

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824-1101

Received 21 March 1986/Accepted 1 August 1986

Direct fermentation of unhydrolyzed potato starch to ethanol by monocultures of an amylolytic fungus, *Aspergillus niger*, and cocultures of *A. niger* and *Saccharomyces cerevisiae* was investigated. Amylolytic activity, rate and amount of starch utilization, and ethanol yields increased several-fold in coculture versus the monoculture due to the synergistic metabolic interactions between the species. Optimal ethanol yields were obtained in the pH range 5 to 6 and amylolytic activity was obtained in the pH range 5 to 8. Ethanol yields were maximal when fermentations were conducted anaerobically. Increasing *S. cerevisiae* inoculum in the coculture from 4 to 12% gave a dramatic increase in the rate of ethanol production, and ethanol yields of >96% of the theoretical maximum were obtained within 2 days of fermentation. These results indicate that simultaneous fermentation of starch to ethanol can be conducted efficiently by using cocultures of the amylolytic fungus *A. niger* and a nonamylolytic sugar fermenter, *S. cerevisiae*.

Large volumes of starchy feedstock such as corn represent an important biomass resource for fuel alcohol production, because the chemical composition and high density of starch, compared to other forms of biomass, facilitates prolonged storage and decreased transportation and pre-treatment costs. Large quantities of starch-rich agroindustrial residues such as potato-processing wastes represent another important resource which could be fermented to yield ethanol. For example, an estimated 3.6×10^9 kg of wet potato-processing wastes is generated annually in the United States (3) which is potentially convertible by fermentation into about 126×10^9 gallons (476.9×10^9 liters) of ethanol. The production of industrial and fuel ethanol from starchy biomass commonly involves a three-step process (7): (i) liquefaction of starch by an endoamylase such as α -amylase; (ii) enzymatic saccharification of the low-molecular-weight liquefaction products (dextrins) to produce glucose; and (iii) fermentation of glucose to ethanol. Commercial amylases (frequently those produced by *Aspergillus* species) are used for liquefaction and saccharification of starch and represent a significant expense in the production of fuel alcohol from starchy materials.

Initial studies aimed at the elimination of the enzymatic liquefaction and saccharification step by using symbiotic coculture of amylolytic and sugar-fermenting organisms have been promising. For example, in the "Symba" process for single-cell protein production from potato-processing wastes, Jarl (4) and Skogman (11) eliminated the enzymatic liquefaction and saccharification step by using a coculture of *Endomycopsis fibuligera* (an amylolytic yeast) and *Candida utilis* (a nonamylolytic sugar utilizer). Lalue and Mattoon (7) suggested the use of *Saccharomyces diastaticus* for direct conversion of manioc starch to ethanol; however, prior treatment of the starch with α -amylase was required for obtaining efficient fermentation of starch. The purpose of this study was to develop and evaluate a simultaneous

single-step system for the enhanced fermentation of potato starch to ethanol by using symbiotic cocultures of *Aspergillus* species, which hydrolyze starch to glucose, and *S. cerevisiae*, which is nonamylolytic but efficiently ferments glucose to ethanol.

MATERIALS AND METHODS

Organisms. Amylase-producing fungi, *A. niger* (NRRL 330), *A. foetidus* (NRRL 337), and *A. awamori* (NRRL 3112), were obtained from the Northern Regional Research Laboratory, U.S. Department of Agriculture, Ill., and were maintained on potato dextrose agar slants (Difco Laboratories, Detroit, Mich.). *S. cerevisiae* (ATCC 26603) was obtained from the American Type Culture Collection, Rockville, Md., and was maintained on slants of sterile YM agar medium which contained, per liter: yeast extract, 3 g; malt extract, 3 g; peptone, 5 g; glucose, 10 g; and agar, 20 g. Commercial dried yeast (*S. cerevisiae* type II) used as inoculum in some experiments (see Fig. 3 and Fig. 4) was purchased from Sigma Chemical Co., St. Louis, Mo. The results were comparable when bulk dried yeast obtained from Diamond-V. Mills Inc. (Cedar Rapids, Iowa) was used.

Potato starch. Potato starch used in this investigation was described previously (3) and was recovered from the wastewater of a potato chip manufacturing plant, air dried, pulverized in a Waring blender, and stored at room temperature. This dried potato starch contained 98.6% (wt/wt) sugar based on total carbohydrate estimation on an acid-hydrolyzed sample (3).

Media. The growth medium used for preparing the fungal inocula contained, in grams per 100 ml: potato starch, 1; peptone, 0.1; malt extract, 0.1; yeast extract, 0.2; magnesium chloride $\cdot 6H_2O$, 0.1; calcium carbonate, 0.2; ammonium phosphate, 0.2; and ferrous sulfate $\cdot 7H_2O$, 0.001.

The fermentation medium used for ethanol production from starch was identical to the growth medium except that the starch concentration varied from 1 to 10 g in different experiments. For testing the effect of pH on fermentation, 1 N HCl or 1 N NaOH was added to this medium to obtain the desired initial pH.

* Corresponding author.

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Preparation of inocula. Fungal inocula were prepared by using slant cultures to inoculate 20 ml of sterile growth medium (see above) contained in 50-ml foam-stoppered Erlenmeyer flasks. The flasks were incubated with shaking (200 rpm) at 30°C for 5 days.

S. cerevisiae inoculum was prepared the same way as the fungal inoculum except that YM broth (pH 5.5) was used (instead of growth medium) and incubation was for 24 h.

Fermentation procedures. Ethanol fermentation was carried out in 500-ml Erlenmeyer flasks containing 200 ml of medium. The flasks were sterilized by autoclaving at 121°C for 30 min and a 5% (vol/vol) inoculum of a given amyolytic fungus or yeast was used, unless otherwise mentioned. In some experiments, varying amounts (4 to 12% wt/vol) of dried *S. cerevisiae* were used as the inoculum. Unless otherwise mentioned cultures were incubated under "limited anaerobic" conditions (see below) with shaking (200 rpm) at 30°C for 7 days.

For studying the effect of aeration on ethanol production, flasks were fitted with foam plugs ("aerobic") or with a one-hole rubber stopper into which a cotton-plugged Pasteur pipette was inserted to vent out CO₂ evolved during the fermentation ("limited aerobic"). Flasks referred to as "anaerobic" were identical to the limited aerobic flasks except that the cotton-plugged end of the glass tube was connected to a latex rubber tube, the other end of which was placed under water in a large glass test tube. Cultures which were first grown under aerobic conditions for 24 h, flushed with N₂ for 2 min, and then incubated under anaerobic conditions are referred to as "anaerobic-N₂" cultures.

Analytical procedures. Samples (10 ml) were collected from a given flask and centrifuged at 4°C for 20 min at 5,000 × g to remove cells, and the supernatant fluid was used for determining ethanol and reducing sugar concentration and amyolytic activity. The theoretical ethanol yield was calculated assuming complete conversion of glucose, obtained from starch hydrolysis, to ethanol, whereby 180 g of glucose (1 mol) yields 92 g of ethanol (2 mol). This value was then used to calculate the percentage of ethanol produced in the experimental flask.

Residual starch concentrations in a 100-μl sample of undiluted culture broth or in a culture broth appropriately diluted with water was determined as glucose by using the phenol-sulfuric acid method (2). The amount of starch in the sample was then calculated by using the formula of Keer (5): glucose (grams/100 ml) × 0.93 = starch (grams/100 ml).

The extracellular amyolytic activity in culture broth was determined by measuring reducing sugar released from starch as described by Lemmel et al. (8) in which the reduction of 3,5-dinitrosalicylic acid to nitroaminosalicylic acid by the reducing sugar in the sample was determined (9). A standard curve for this colorimetric assay was constructed, using glucose as the standard. One unit of amyolytic activity was defined as the amount of enzyme in 1 ml that liberates 1 μmol of reducing sugar from starch in 3 min.

Mean values of three separate experiments and for two replicate flasks per experiment are presented. Variations of 4 to 7 and ≤5% were seen between individual experiments and replicate flasks, respectively.

Cell biomass. The cell biomass was determined by harvesting cells by centrifugation, drying them at 70°C under vacuum to a constant weight, and expressing the dry weight as grams per 100 ml of growth medium.

Ethanol concentration. Ethanol concentration was determined by a gas chromatograph (Varian Aerograph series

2400; Varian Co., Sunnyvale, Calif.) equipped with a stainless-steel column packed with Chromosorb W (acid washed and 80/100 mesh; Supelco Co., Bellefonte, Pa.) and a flame ionization detector. The carrier gas was N₂ (30 ml/min) and the column temperature was set at 80°C.

RESULTS AND DISCUSSION

Utilization of starch by monocultures and cocultures. Recent studies showed that amylase activity in cultures of different *Aspergillus* species is subject to severe feedback inhibition by glucose derived from starch hydrolysis (C. A. Reddy and M. M. Abouzied, *Enzyme Microb. Technol.*, in press). Therefore, the objective of the first series of experiments was to test the hypothesis that coculturing of an efficient sugar fermentor such as *S. cerevisiae* with an *Aspergillus* species in a starch medium would prevent accumulation of inhibitory concentrations of reducing sugar and that this would result in an enhancement of the amyolytic activity, the amount of starch metabolized, and the total ethanol yield. The results showed that starch utilization, amyolytic activity, and ethanol yields are low in monocultures of *A. niger*, *A. foetidus*, and *A. awamori*, whereas in cocultures of these organisms with *S. cerevisiae* there was a dramatic increase in amyolytic activity and ethanol production (Table 1). Starch utilization of up to 94.8% observed with cocultures was much higher than that observed in monocultures. The results also show that cocultures of *Aspergillus* sp. and *S. cerevisiae* could simultaneously ferment starch to ethanol.

Incomplete utilization of starch by monocultures of *Aspergillus* species may be due to lack of enough oxygen or to feedback inhibition of amylase activity by glucose released from starch hydrolysis, as recently reported (Reddy and Abouzied, *Enzyme Microb. Technol.* in press), or both.

Coculture of *A. niger* and *S. cerevisiae* was selected for further study because *A. niger* consistently gave higher ethanol yields than the other cocultures. When *A. niger* was grown alone and in coculture with *S. cerevisiae* in media containing different concentrations of starch and incubated under limited aerobic conditions (Table 2), starch utilization by mono- and cocultures was comparable, but ethanol production by the coculture was substantially higher at each of the substrate concentrations tested. Furthermore, ethanol

TABLE 1. Comparison of starch metabolism parameters in monocultures and cocultures of *Aspergillus* species and *S. cerevisiae*^a

Fungus	<i>S. cerevisiae</i>	Ethanol (g/100 ml)	Residual starch (g/100 ml)	Amyolytic activity (U/ml)
<i>A. niger</i>	—	0.27	1.4	ND
<i>A. niger</i>	+	1.9	0.26	10.6
<i>A. foetidus</i>	—	0.40	1.86	ND
<i>A. foetidus</i>	+	1.38	0.52	10.6
<i>A. awamori</i>	—	0.23	2.1	ND
<i>A. awamori</i>	+	1.23	0.82	10.6

^a Experiments were conducted in 500-ml Erlenmeyer flasks containing 200 ml of 5% starch medium and were incubated under limited aerobic conditions, with shaking, for 7 days at 30°C (see Materials and Methods). Mean values of at least three separate experiments and for two identical flasks per experiment are presented. Variation of 4 to 7% was seen between individual experiments. ND, No detectable level of activity. Only trace amounts of growth and ethanol production were seen in negative control flasks inoculated with *S. cerevisiae* only.

TABLE 2. Utilization of different concentrations of starch by monocultures and cocultures of *A. niger* and *S. cerevisiae*^a

Starch	g/100 ml of medium					
	<i>A. niger</i>			<i>A. niger</i> + <i>S. cerevisiae</i>		
	Starch consumed	Cell biomass	Ethanol	Starch consumed	Cell biomass	Ethanol
1	0.9	1.0	0.17	0.96	0.9	0.18
2	1.9	1.5	0.17	0.96	1.0	0.48
3	2.5	1.8	0.29	2.9	1.1	0.70
4	3.7	2.1	0.29	3.8	1.4	1.03
5	4.2	2.7	0.30	4.8	1.6	1.33

^a Other experimental conditions were as described in footnote a, Table 1.

production in the coculture was proportional to the starch utilized.

Higher cell mass was produced in monoculture than in cocultures, suggesting that substantially more carbon is used for cell production in monoculture, whereas in the coculture most of the substrate carbon is utilized for ethanol production. *S. cerevisiae* was the dominant organism in coculture.

Time course of starch utilization, reducing sugar, ethanol concentrations, and amylolytic activity in cocultures of *A. niger* and *S. cerevisiae* in 5% starch medium were then determined (Fig. 1). Concomitant with starch hydrolysis there was a rapid increase in reducing sugar concentration initially. During the rest of the fermentation (days 2 to 7) rapid fermentation of sugar to ethanol by *S. cerevisiae* kept the sugar concentration low enough to prevent feedback inhibition of amylolytic activity previously observed in monocultures of *A. niger* (Reddy and Abouzied, in press).

Effect of pH. The effect of initial pH on direct fermentation of starch to ethanol by cocultures of *A. niger* and *S. cerevisiae* was determined by monitoring amylolytic activity and ethanol concentration (Fig. 2). Ethanol production was optimal in the pH range 5 to 6. However, amylolytic activity

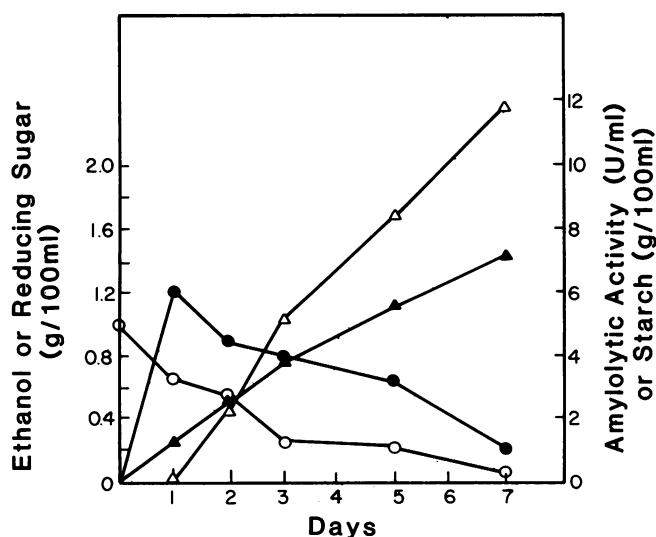


FIG. 1. Time course of starch fermentation by cocultures of *A. niger* and *S. cerevisiae*. This experiment was conducted in seven Erlenmeyer flasks containing 5% (wt/vol) starch medium under limited aerobic conditions (see Materials and Methods) and incubated with shaking at 30°C. At the end of each day of incubation, the contents of one flask were analyzed. Symbols: ○, starch; ●, reducing sugar; △, amylolytic activity; ▲, ethanol.

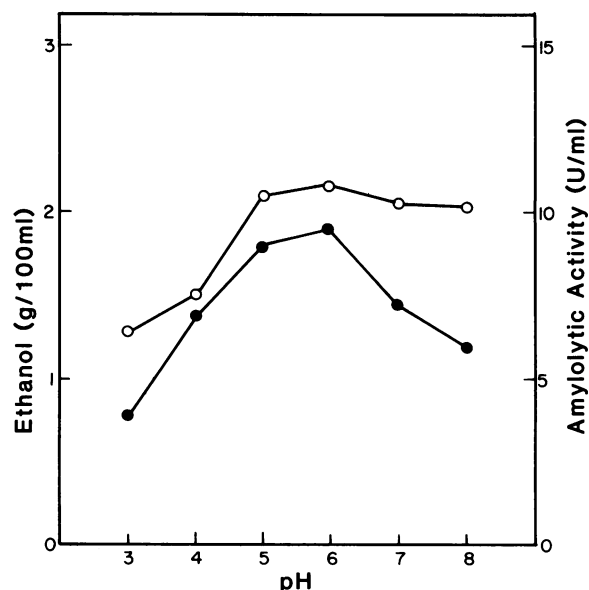


FIG. 2. Effect of initial pH on amylase activity and ethanol yield by cocultures of *A. niger* and *S. cerevisiae*. Other experimental conditions were the same as in the legend to Fig. 1. Symbols: ○, amylolytic activity; ●, ethanol.

had a much broader optimal pH range of 5 to 8. The average optimal pH of 5.5 was used in most of the experiments.

Effect of aeration. Aeration is known to have a profound effect on yeast alcohol fermentation (6). Yeasts ferment sugars to ethanol primarily by way of the Embden-Myerhof pathway. A small concentration of oxygen must be provided to the fermenting yeast as it is necessary for the biosynthesis of certain polyunsaturated fats and lipids. Excess oxygen in the fermentation medium, on the other hand, will promote respiration and cell growth at the expense of ethanol productivity (6). Among the various treatments tested in this experiment, the anaerobic and anaerobic-N₂ incubations appeared to be optimal for ethanol production by coculture of *A. niger* and *S. cerevisiae*. Fermentation under aerobic conditions resulted in the least amount of ethanol production but gave the highest biomass yield compared with the other incubation conditions tried (Table 3).

Effect of starch and yeast concentration. Kinetics of ethanol production at increasing concentrations of starch (5 to 10%), using cocultures of *A. niger* and *S. cerevisiae*, was then determined. *A. niger* inoculum was the same as in other experiments (5%, vol/vol), but 6% (wt/vol) dry *S. cerevisiae* inoculum was used. The results showed that ethanol yields were comparable at each of the starch concentrations tested (Fig. 3). We then tested the effect of increasing the level of

TABLE 3. Effect of aeration conditions on fermentation of starch by cocultures of *A. niger* and *S. cerevisiae*^a

Treatment ^b	g/100 ml of medium		
	Starch utilized	Ethanol	Cell biomass
Aerobic	4.8	0.78	1.53
Limited aerobic	4.6	1.35	0.90
Anaerobic	4.7	1.59	0.77
Anaerobic-N ₂	4.7	1.59	0.93

^a Other experimental conditions were as described in footnote a, Table 1.

^b See Materials and Methods for a description of the terms.

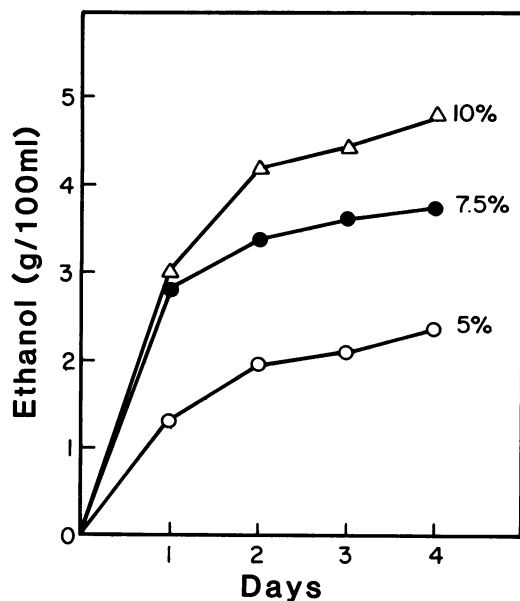


FIG. 3. Effect of starch concentration on ethanol production in cocultures of *A. niger* and *S. cerevisiae*. Other conditions were the same as in the legend to Fig. 1, except that 6% (wt/vol) dry *S. cerevisiae* cells (see Materials and Methods) were used.

yeast inoculum in the coculture on the length of fermentation and ethanol yield. Furthermore, since it would be more desirable for industrial applications to be able to utilize high substrate concentrations for fermentation, 10% (wt/vol) starch was used in this experiment. The results (Fig. 4) showed that as the concentration of yeast inoculum in-

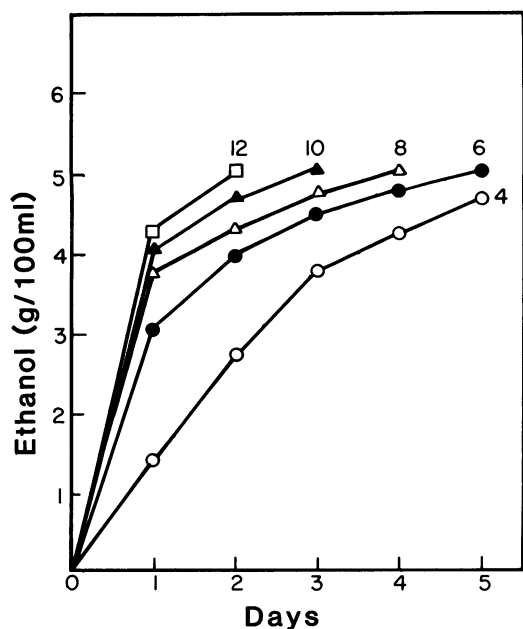


FIG. 4. Effect of varying yeast concentration on ethanol production in cocultures of *S. cerevisiae* and *A. niger*. Experimental conditions were the same as in the legend to Fig. 1, except that the starch concentration was raised to 10% and the yeast inoculum consisted of 4 to 12 g of dry *S. cerevisiae* cells (see Materials and Methods) per 100 ml of medium.

creased, the time required for the completion of fermentation decreased dramatically. For example, using a 12% yeast inoculum, starch was completely utilized in 2 days, and the efficiency of starch conversion to ethanol was 96% of the theoretical maximum expected. These data indicate that in *A. niger* plus *S. cerevisiae* coculture fermentations containing high levels of starch (10%, wt/vol), the release of reducing sugar from starch by the amylolytic enzyme system of *A. niger* is not the limiting step. Instead, the rate of fermentation of sugar by *S. cerevisiae* appears to be the limiting factor. It is also evident from these data that at least ethanol concentrations up to 5% are not inhibitory to *A. niger*. The above results are in agreement with those of Nagodawithana and Steinkraus (10) and Abouzied and Steinkraus (1), who showed that increasing the level of yeast inoculum greatly decreases the fermentation time, especially when high substrate concentrations are used.

It is noteworthy that when 4 to 12% yeast inocula are used the final ethanol yields are comparable; it is only the length of fermentation that is markedly affected. Since fermentations that are rapid and yield high concentration of ethanol are economically desirable, there is need for further investigations to develop an efficient continuous process for the fermentative conversion of starch to ethanol by using synergistic cocultures such as those employed in this study.

The results of this investigation clearly show that simultaneous fermentation of potato starch to ethanol by a mixture of starch-digesting fungus and a non-starch-digesting, sugar-fermenting organism such as *Saccharomyces* is feasible. The efficiency of starch conversion to ethanol was >96% of the theoretical maximum expected. Use of such a synergistic combination of organisms allows elimination of the enzymatic starch hydrolysis step as currently used in many commercial processes for ethanol production from starchy biomass, thereby significantly improving the economy of starch fermentation to ethanol. To the best of our knowledge, this is the first description of the use of synergistic cocultures for the simultaneous conversion of starch to ethanol.

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