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Comparison of fumaric acid production by *Rhizopus oryzae* using different neutralizing agents

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Abstract Fumaric acid fermentation in a 10-L bubble column fermenter using different neutralizing agents [$CaCO_3$, $Ca(OH)_2$, $NaHCO_3$] by *Rhizopus oryzae* ATCC 20344 was examined. It was found that in the fermentation using $CaCO_3$ as the neutralizing agent the highest fumaric acid weight yield and volumetric productivity were obtained, 53.4% and 1.03 $g/L{\times}h^{-1}$ respectively. In the $NaHCO_3$ case, the fumaric acid weight yield and volumetric productivity were 33.7% and 0.69 $g/L{\times}h^{-1}$, respectively, much lower than the $CaCO_3$ case. However, the $NaHCO_3$ alternative has advantages of cell reuse and simple downstream processing because of the high solubility of sodium fumarate. These advantages may offset the disadvantages of using $NaHCO_3$ as the neutralizing agent, and the overall fumaric acid weight yield and volumetric productivity will increase.

Introduction

Fumaric acid has extensive applications in various industries, such as in the manufacture of sizing resins for the paper industry, as an acidulant in food industry, and as a promising candidate in the manufacture of polymer [9].

Fumaric acid can be produced by fungal fermentation. Especially, the genus *Rhizopus* within the order *Mucorales* has been studied by many researchers [1, 2, 3, 4, 5, 6, 7, 8, 10]. It was found that organisms in the genus *Rhizopus* could not tolerate high acidity and that both growth and fumarate production were greatly enhanced by the presence of neutralizing agents [7, 8]. Limestone ($CaCO_3$) has been widely used for this purpose, but has the disadvantage that the solubility of calcium fumarate is not as high as desirable for building up economically feasible concentrations of this salt. At 35°C, its solubility is approximately 2.5%. As the fermentation process proceeds the whole mass sets to a solid gel. Therefore, it is attempted to use different neutralizing agents to provide higher fumarate solubility, while maintaining fumarate production yield and reducing the production cost.

The ability of *Rhizopus oryzae* to produce fumaric acid aerobically in different reactors has been studied [1, 2, 3]. In this report, fumaric acid fermentation in a 10-L bubble column fermenter by *Rhizopus oryzae* ATCC 20344 using different neutralizing agents was examined and results were compared and discussed.

Materials and methods

Microorganism and inoculum

Rhizopus oryzae ATCC 20344 was used in this work. The fungus grew and formed spores on YMP agar plates at 27°C for 8 days, and was maintained at 4°C. To prepare inoculum, agar plates containing sporulated fungi were washed with sterile water to obtain a spore suspension. For each experiment, the spore suspension was inoculated into three 2.0-L Erlenmeyer flasks containing 800 mL cultivation medium. The cultivation medium consisted of 30 g glucose, 3 g urea, 1 g KH_2PO_4, 0.1 g $ZnSO_4{\cdot}7H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$. Incubation was carried out at 35°C and 200 rpm in the incubator-shaker (Model G-24, New Brunswick Scientific, N.J.) for 18 h.

Neutralizing agents

Three different neutralizing agents ($CaCO_3$, $Ca(OH)_2$, $NaHCO_3$) were chosen in this experiment to examine the effect of neutralizing agents on fumaric acid fermentation by *R. oryzae* ATCC 20344. Most previous investigators of fumaric acid production using neutralizing agent by *Rhizopus* species have used calcium carbonate [6, 7, 8, 10]. $Ca(OH)_2$ was chosen because the market price of $Ca(OH)_2$ is much cheaper than $CaCO_3$. $NaHCO_3$ was chosen as the neutralizing agent because of the higher solubility of sodium fumarate (22%, w/v).

Fermentation procedure

A 10-L bubble column fermenter was employed in this experiment. A perforated ring (pore size 1–2 mm) was inserted into the bottom of the fermenter as the air sparger.

The culture after 18 h of preculture was transferred aseptically into the sterilized bubble column fermenter with addition of sterilized glucose solution only. The fermentation was operated at an aeration rate of 1.5 vvm and 32°C. After 12 h of incubation in the bubble column fermenter, the pH of the broth dropped from about 5.0 to 3.0. During this period, the germinated spores grew into more

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Table 1. Comparison of fumaric acid fermentation by *R. oryzae* ATCC 20344 in the 10-L bubble column fermenter using different neutralizing agents

Parameter	Neutralizing agent		
	$CaCO_3$	$NaHCO_3$	$Ca(OH)_2$
pH controlled	5.5	5.5–5.6	5.5–5.6
Initial glucose (g/L)	95	98	74
Residual glucose (g/L)	25.3	0	11.6
Fermentation time (h)	36	48	42
Final fumaric acid (g/L)	37.2	33.0	10.8
Final ethanol (g/L)	10.5	13.3	10.4
Malic acid (g/L)	0	0	5.2
Fumaric acid weight yield (%)[a]	53.4	33.7	17.3
Volumetric fumaric acid productivity ($g/L\times h^{-1}$)	1.03	0.69	0.26

[a]Fumaric acid weight yield (%): gram of fumaric acid produced/gram of glucose consumed

or less uniform-size elliptical pellets (pellet size of 1–1.5 mm). Then, the neutralizing agent was pumped into the fermenter to maintain the pH of the broth around 5.5. When $CaCO_3$ was used as the neutralizing agent, excessive $CaCO_3$ was added periodically to maintain the pH of the broth at the natural pH of $CaCO_3$ (pH=5.5). When either $NaHCO_3$ or $Ca(OH)_2$ was chosen as the neutralizing agents, a pH probe was employed to measure the pH online and pump the neutralizing agents on demand to maintain the pH at the desirable level (pH=5.5–5.6).

Analytical methods

A high-performance liquid chromatograph (Model L-6200A) with a refractive index detector (Model L-3350 RI), an automatic injector (Model AS-4000) and an integrator (Model D-2500) (Hitachi Instrument, Japan) was used to analyze glucose, fumaric acid, and byproduct concentrations. The mobile phase used was 0.005 M H_2SO_4 at a flow rate of 0.8 mL/min through a BioRad Aminex HPX-87H ion exclusion column (BioRad Laboratories, Hercules, Calif.) at 60°C.

Because of the low solubility of calcium fumarate, fumarate precipitated in the fermentation broth. The final culture broth was diluted by addition of hydrochloric acid and distilled water to neutralize excessive $CaCO_3$ and dissolve the fumarate before the samples were taken.

Results and discussion

The comparison of fumaric acid fermentation in the 10-L bubble column using different neutralizing agents was summarized in Table 1. It was found that in the fermentation using $CaCO_3$ as the neutralizing agent, the highest fumaric acid weight yield and volumetric productivity were obtained, 53.4% and 1.03 $g/L\times h^{-1}$, respectively. In the experiment, the fermentation was ended when calcium fumarate precipitation was observed. Precipitation of calcium fumarate in the fermentation broth results in higher viscosity of the medium and more resistance for substrate and oxygen diffusion into the pellets. Therefore, the presence of insoluble crystals increases power consumption during the later phase of fermentation. Also, to recover fumaric acid mineral acid which yields a soluble inorganic calcium salt, such as HCl or HNO_3, and heat are used to remove pellets cells from precipitated calcium fumarate; then fumaric acid is crystallized from the fermentation broth. However, using $CaCO_3$ as the neutralizing agent in the fumaric acid fermentation has the advantage of elimination of a pH control system for base addition.

In the fermentation using $Ca(OH)_2$ as the neutralizing agent, the fumaric acid weight yield and volumetric productivity are 3.2 times and 2.5 times lower than those in the $CaCO_3$ case. This difference probably results from more dissolved CO_2 being available to the organism in the $CaCO_3$ case. This result is consistent with the theory that CO_2 fixation is necessary for production of fumaric acid by *R. oryzae*. However, though $Ca(OH)_2$ is much cheaper than $CaCO_3$, it is still not a good substitute for $CaCO_3$.

In the $NaHCO_3$ case, the fumaric acid weight yield and volumetric productivity were 33.7% and 0.69 $g/L\times h^{-1}$, respectively, which is much lower than the $CaCO_3$ case. This is probably due to the effect of sodium in the medium on the fermentation mechanism. Because of the high solubility of sodium fumarate, the pellet cells can be easily separated from the fermentation broth without heat. And these cells can be recycled for use in the next fermentation. This will increase the overall fumaric acid weight and productivity. Also, because of the low solubility of fumaric acid (0.7%, w/v), it can be easily recovered by acidifying the salt. Therefore, using $NaHCO_3$ as the neutralizing agent simplifies downstream processing by requiring less equipment and no heat. These advantages may offset the two disadvantages of using $NaHCO_3$ as the neutralizing agent: a slightly higher cost of $NaHCO_3$ than $CaCO_3$ and a lower fumaric acid productivity for the fermentation using $NaHCO_3$ instead of $CaCO_3$.

Conclusion

Results of fumaric acid fermentation by *R. oryzae* ATCC 20344 in the 10-L bubble column fermenter using different neutralizing agents($CaCO_3$, $Ca(OH)_2$, $NaHCO_3$) showed that in the fermentation using $CaCO_3$ as the neutralizing agent, the highest fumaric acid weight yield and volumetric productivity were obtained. In the fermentation using $NaHCO_3$ as the neutralizing agent, there are the advantages of reuse of cells and simpler downstream separation; these advantages may offset the disadvantages of lower fumaric acid weight yield and volumetric productivity and make this process more economic than the fermentation using $CaCO_3$ as the neutralizing agent. Therefore, $NaHCO_3$ may be an alternative neutralizing agent to replace $CaCO_3$ in fumaric acid fermentation. Further study to improve the fumaric acid production using $NaHCO_3$ as the neutralizing agent is necessary.

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