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Conversion of β-Methylbutyric Acid to β-Hydroxy-β-Methylbutyric Acid by *Galactomyces reessii*

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β-Hydroxy-β-methylbutyric acid (HMB) has been shown to increase strength and lean mass gains in humans undergoing resistance-exercise training. HMB is currently marketed as a calcium salt of HMB, and thus, environmentally sound and inexpensive methods of manufacture are being sought. This study investigates the microbial conversion of β-methylbutyric acid (MBA) to HMB by cultures of *Galactomyces reessii*. Optimal concentrations of MBA were in the range of 5 to 20 g/liter for HMB production. Preliminary shake flask experiments indicated that HMB yields were sensitive to dissolved oxygen levels and that cell growth decreased significantly as MBA concentrations increased. Degradation of HMB was faster at acidic pH, and pH 7.0 was optimal for HMB production. Resting cells obtained from media without MBA could efficiently convert MBA to HMB. Thus, a two-step, fed-batch fermentation procedure in which biomass was first produced, followed by coaddition of MBA and glucose, while dissolved oxygen was maintained at 20% of saturation, was designed. A maximum HMB concentration of 38 g/liter was obtained after 136 h, and the molar conversion yield was more than 0.50 mol of HMB/mol of MBA during the fermentation.

β-Hydroxy-β-methylbutyric acid (HMB) is produced from leucine via transamination to α-ketoisocaproate in both humans and animals (10–12). HMB has been shown to improve animal growth and health in numerous studies (7, 8, 13). Economical means for the manufacture of HMB are necessary, however, before these benefits can be exploited for food-producing animals. Recently, calcium HMB has been shown to increase strength and lean mass gains in resistance-exercise training in humans (9). These studies have stimulated interest in developing methods for the commercial production of HMB. Currently, a chemical synthetic route is used to produce HMB for this purpose. However, as the commercial potential for the use of HMB increases, it will be important to develop environmentally sound and inexpensive methods of HMB production.

HMB can be chemically synthesized by sodium hypochlorite-catalyzed oxidation of diacetone alcohol (1). Syntheses like these employ reactive chemicals and often generate environmentally undesirable by-products and residues. An efficient microbial synthesis of HMB which could produce the compound under mild reaction conditions without the need for either drastic reactions or reagents would provide obvious advantages. Microbiological syntheses of a variety of β-hydroxycarboxylic acids were reported by Hasegawa et al. (2, 3). Among these, microbial transformation of isobutyric acid into D-β-hydroxyisobutyric acid has been successfully used for the industrial-scale synthesis of the antihypertensive agent Captopril (2). Hasegawa et al. (3) showed that cultures of *Galactomyces reessii* (formerly *Endomyces reessii*) could convert β-methylbutyric acid (MBA) to HMB in low yield. However, no further experiments designed to develop an efficient HMB production process have been reported.

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Lee et al. (5, 6) recently reported that high levels of D-β-hydroxyisobutyric acid could be obtained by microbial transformation of either isobutyric acid or methacrylic acid using a fed-batch cultivation system with *Candida rugosa*. Since MBA is very similar to D-β-hydroxyisobutyric acid in structure, we attempted to use this experience in developing a relatively high yielding process for the biotransformation of MBA to HMB.

This paper describes studies designed to optimize stirred jar fermentation cultures of *G. reesii* and conversions of MBA to HMB by controlling pH, dissolved oxygen, and substrate concentrations to achieve a titer of nearly 40 g of HMB/liter.

MATERIALS AND METHODS

Microorganism and culture media. *G. reessii* CBS 179.60 was used throughout these experiments. Luria broth (LB) contained (per liter) 10 g of yeast extract, 5 g of tryptone, 10 g of NaCl, and 10 g of glucose. The basal fermentation (GI) medium contained (per liter) 40 g of glucose, 13 g of $(NH_4)_2HPO_4$, 7 g of KH_2PO_4, 0.8 g of $MgSO_4 \cdot 7H_2O$, 3 g of yeast extract, 1 g of NaCl, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.05 g of $CaCl_2 \cdot 2H_2O$, and 10 ml of trace element solution (per liter of 1 N HCl, 0.3 g of H_3BO_3, 0.2 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, 30 mg of $MnCl_2 \cdot 4H_2O$, 30 mg of $Na_2MoO_4 \cdot 2H_2O$, 20 mg of $NiCl_2 \cdot 6H_2O$, and 10 mg of $CuSO_4 \cdot 5H_2O$). Predetermined amounts of MBA or HMB were added to GI medium, and the pH was adjusted to 7.2 with an 8 N NaOH–8 N KOH mixture. For fed-batch operations, two different feed solutions were used: (i) a glucose feed solution prepared by dissolving 700 g of glucose, 15 g of KH_2PO_4, 15 g of $Na_2HPO_4 \cdot 7H_2O$, and 5 g of $MgSO_4 \cdot 7H_2O$ in 1 liter of distilled water and (ii) an MBA feed solution prepared by mixing MBA, 8 N NaOH, 8 N KOH, and NH_4OH (28%) (8:1:1:1).

Flask cultures. For flask cultures, a loop of the microorganism was inoculated into 25 ml of LB medium held in 125-ml, conical-shaped, stainless steel-capped, DeLong culture flasks and cultivated with shaking at 250 rpm for 24 h at 30°C on a rotary shaker. A 2-ml suspension of cells grown in LB was used to inoculate 25 ml of GI medium containing either MBA or HMB in 125-ml DeLong flasks, which were incubated as described above. To examine the possible effects of dissolved oxygen on MBA conversion yields, different relative volumes of medium were used in shake flasks.

A two-stage culture technique was also used to examine the inducibility of enzymes involved in HMB synthesis and the effects of reaction medium on HMB production. The microorganism was first grown at 30°C for 24 h in either LB medium or LB medium containing 0.5% (wt/vol) MBA. Then, cells (60 or 100 mg [dry weight]) harvested by centrifugation at 8,000 × *g* for 10 min were suspended in 25 ml of 0.1 M phosphate buffer (pH 7.0) containing 1% (wt/vol) MBA. Supplements to the cell suspensions included glucose, $MgSO_4 \cdot 7H_2O$, and trace elements as described below.

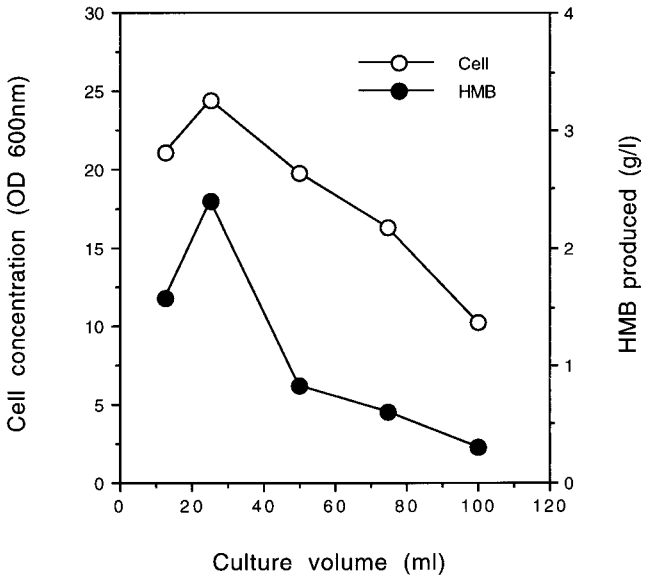

FIG. 1. Effect of culture volume in 125-ml flasks on cell growth and conversions of MBA to HMB by *G. reessii*. Cells were cultivated with shaking at 250 rpm for 55 h at 30°C in GI medium containing 1% (wt/vol) MBA.

All flask cultures were prepared and analyzed in triplicate. Results are expressed as the mean values of three determinations within a deviation of not more than 10%.

Fed-batch conversions of MBA to HMB in fermentors. Fed-batch cultivations of *G. reessii* were done in 10-liter glass jars by using a Bioflo 3000 fermentor (New Brunswick Scientific Co., Edison, N.J.) equipped with a polarographic Ingold dissolved oxygen (DO) probe and pH controllers. Seed culture (400 ml) grown in LB at 30°C for 30 h in shake flasks was used to inoculate the fermentor containing 3.6 liters of GI medium. After cells grew to a concentration of 23 g/liter (dry weight), MBA feed solution was supplied to maintain MBA in the culture broth at a concentration of less than 20 g/liter (by gas chromatography [GC] analysis). In addition, glucose solution was fed into the fermentor at a constant flow rate of 5 g/h. The pH value was controlled at 6.5 during cell growth with NH_4OH (28%) and then shifted to pH 7.0 in the HMB production step after MBA was added. The DO concentration was maintained at 20% saturation by automatically controlling both agitation speed and aeration rate.

Analytical methods. Cell growth was monitored by measuring the optical density of the culture broth at 600 nm (OD_{600}) and by measuring the dry weight of cells. Glucose concentrations were determined with a YSI model 2700 glucose analyzer (Yellow Springs Instruments, Yellow Springs, Ohio). Concentrations of MBA and HMB were determined by GC above an Alltech AT-1 column (Alltech, Deerfield, Ill.) with a Shimadzu (Kyoto, Japan) model 14A GC. For GC analysis, 1-ml samples of culture broth were mixed with 2 ml of methanol containing 3% sulfuric acid. Samples were heated at 90°C for 3 h, and the methyl esters formed were extracted with 2 ml of chloroform. This method has been extensively used with β-hydroxy-carboxylic acids, which are similar to MBA and HMB (5, 6). MBA was also analyzed without derivatization by high performance liquid chromatography in a C_{18} column (4.6 by 150 mm) with the sample eluted at 1.0 ml/min with $NH_4H_2PO_4$ (5 mM) and adjusted to pH 2.4 with phosphoric acid.

RESULTS

Effect of aeration and culture pH on HMB production and consumption. To examine the effects of volumes of culture on cell growth and HMB production, *G. reessii* was cultivated in 125-ml DeLong flasks containing various volumes of GI medium. As shown in Fig. 1, concentrations of cells (OD_{600} of 25) and HMB (2.40 g/liter) were highest in flasks containing 25 ml of medium. As culture volumes were increased from 25 to 100 ml, both cell growth and HMB production decreased. Smaller culture volumes (12.5 ml) gave lower HMB yields (1.57 g/liter). These results suggested that HMB synthesis was sensitive to the DO concentration.

The effects of pH on cell growth and HMB production were also examined by cultivating *G. reessii* in GI medium adjusted to different initial pH values in flask cultures. Both cell growth and HMB production showed the same patterns over the pH range examined, and the highest concentrations of cells (OD_{600} of 28.2) and HMB (2.50 g/liter) were obtained at an initial pH value of 6.7 (Fig. 2a). To examine the effect of pH on HMB degradation, *G. reesii* was grown in GI medium containing 1% (wt/vol) HMB at different initial pH values. The results in Fig. 2b show that HMB was apparently degraded at a higher rate in media at pH 7 and lower. On the other hand, the culture grew best at slightly acidic pH.

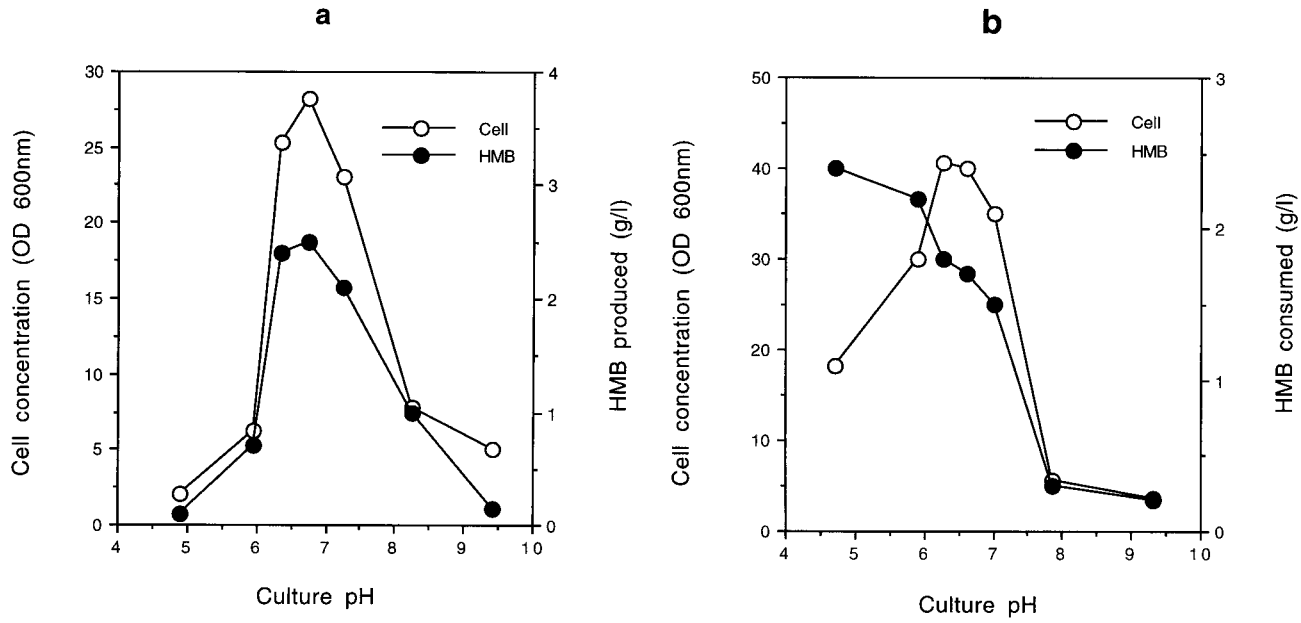

FIG. 2. Effect of culture pH on conversion of MBA to HMB (a) and HMB degradation (b). Cells were cultivated for 55 h at 30°C in GI medium containing 1% (wt/vol) of either MBA or HMB.

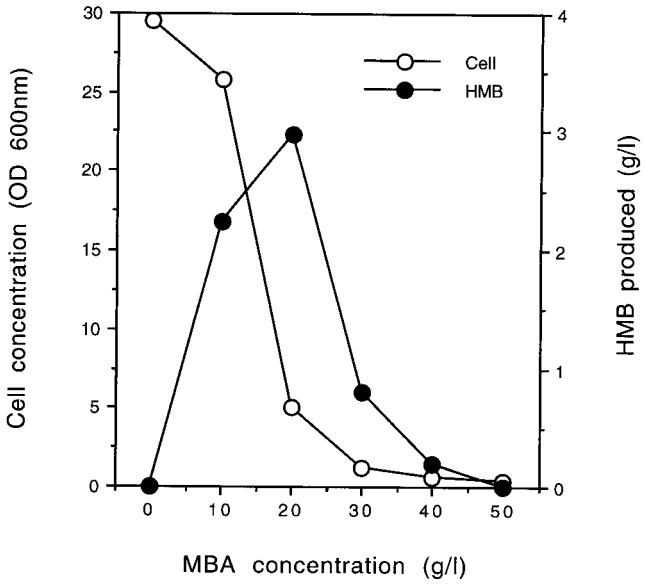

FIG. 3. Effect of MBA concentrations on cell growth and conversion of MBA to HMB. Cells were cultivated for 50 h at 30°C in GI medium containing different concentrations of MBA.

Effect of substrate concentration on HMB yield. To establish optimal concentrations of MBA for HMB production, *G. reessii* was cultivated in flask cultures in GI medium containing different concentrations of MBA. Cell growth decreased as MBA concentrations in the medium increased. Significant inhibition of *G. reessii* cell growth was observed at MBA concentrations of more than 10 g/liter (Fig. 3). However, the highest concentration of HMB (2.95 g/liter) was obtained at an MBA concentration of 20 g/liter in spite of relatively low cell yields. These results indicated that MBA should be maintained at a concentration of less than 20 g/liter in fed-batch fermentations.

Comparison of cells grown with or without MBA for HMB production. The results in Fig. 3 shows that MBA was inhibitory to cell growth. A two-stage culture technique was employed to examine whether cells grown in medium without MBA could efficiently produce HMB. Cultures of *G. reessii* were grown in either LB or LB plus MBA medium, and cells were harvested by centrifugation and resuspended in phosphate buffer containing MBA. Cells obtained from LB plus MBA medium consumed MBA faster than did cells grown in LB medium alone (Fig. 4a). However, HMB production by cells grown in LB medium was comparable to that obtained with the cells grown in LB plus MBA medium (Fig. 4b). Molar conversion yields of HMB from MBA in cells from LB medium were twice as high as those in cells from LB plus MBA medium (Fig. 4c).

Effects of glucose, magnesium, and trace elements on MBA conversion to HMB. *G. reesii* cells were grown in LB medium without MBA, harvested, and resuspended in phosphate buffer containing glucose, magnesium, or trace elements. Buffers supplemented with either only MBA or MBA plus Mg^{2+} gave low yields of HMB even though MBA consumption was high (Table 1). The highest yield of HMB (2.09 g/liter) was obtained in buffer containing both glucose and magnesium sulfate. Under these conditions, MBA consumption was low, resulting in a higher molar conversion yield of 50.2% HMB from MBA. The addition of a trace element mixture had no apparent effect on

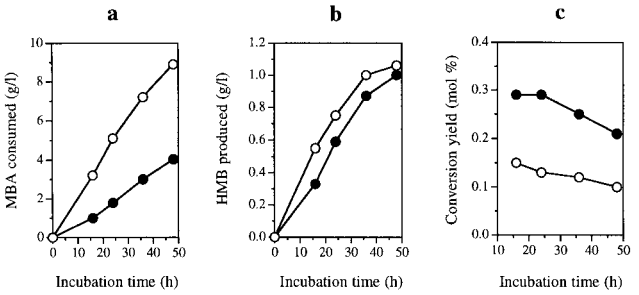

FIG. 4. Comparison of cells grown in LB medium with MBA (○) or without MBA (●) for their abilities to convert MBA to HMB. Cells were first grown at 30°C for 24 h in LB medium with or without MBA. Washed cells (60 mg [dry weight]) were then transferred to 25 ml of 0.1 M phosphate (pH 7.0) buffer and incubated at 30°C on a rotary shaker. Phosphate buffers contained $MgSO_4 \cdot 7H_2O$, 0.8 g/liter; glucose, 20 g/liter; trace elements, 10 ml/liter; and MBA, 10 g/liter.

HMB synthesis. The addition of glucose increased HMB yield and decreased HMB consumption by *G. reessii* cells. HMB production (1.08 g/liter) in 50 ml of buffer plus glucose was almost half the amount of HMB (2.09 g/liter) produced in 25 ml of reaction medium, while the HMB conversion yield was at the same level (51.0 mol%). From these results, we concluded that both the addition of glucose and a sufficient oxygen supply were necessary for HMB production in high yield.

Synthesis of HMB by a two-step, fed-batch fermentation. *G. reesii* cells were first grown at pH 6.5 with nitrogen supplied as 28% ammonium hydroxide for 19 h, after which the MBA addition was begun. MBA concentrations were kept below 20 g/liter, and glucose was added to suppress HMB degradation. The results in Fig. 5 show the time course for culture growth, the concentrations of HMB and MBA, the molar conversion yield, and the specific production rate (qP) during the fed-batch fermentation. The culture reached a cell concentration of 23 g/liter after 19 h of cultivation. When MBA was added, cell growth decreased and then stopped when MBA concentrations were more than 5 g/liter. On the other hand, HMB concentrations continued to increase from the onset of the addition of MBA, and a maximum titer of HMB (38 g/liter) was obtained after 136 h. The conversion yield of HMB from MBA was more than 0.50 mol of HMB/mol of MBA during the

TABLE 1. Effect of incubation buffer composition on production and degradation of HMB by *G. reessii*[a]

Incubation buffer	MBA or HMB consumed (g/liter)	HMB produced (g/liter)	Conversion yield (mol%)
MBA	7.24	1.76	21.0
MBA + Mg	7.87	1.75	19.2
MBA + Mg + Glu	3.60	2.09	50.2
MBA + Mg + Glu + TE	3.96	1.98	43.2
MBA + Mg + Glu (50 ml)[b]	1.83	1.08	51.0
HMB + Mg	3.38		
HMB + Mg + Glu	0.64		

[a] Cells (100 mg [dry weight]) from LB (24 h) were suspended in 25 ml of 100 mM phosphate buffer (pH 7.0) containing either 1% (wt/vol) MBA or HMB. The following components were added at the concentrations indicated: $MgSO_4 \cdot 7H_2O$, 0.8 g/liter; glucose (Glu), 20 g/liter; and trace element (TE), 10 ml/liter. After the culture pH was adjusted to 7.0, incubations were carried out in triplicate in 125-ml DeLong flasks containing 25 ml of cell suspension mixture at 30°C with shaking for 48 h.

[b] Reaction was carried out in 125-ml DeLong flasks containing double the usual volume (50 ml) of cell suspension mixture.

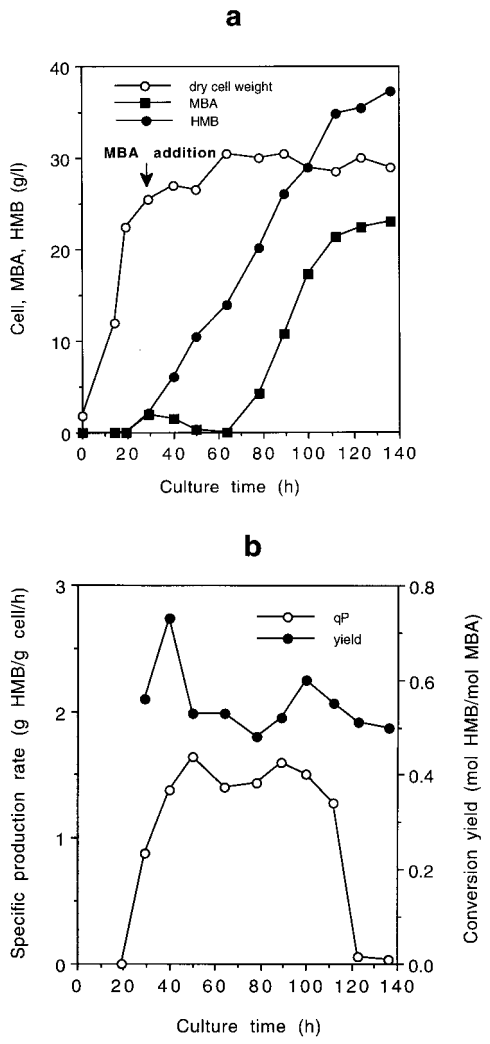

FIG. 5. Conversion of MBA to HMB by a two-step, fed-batch fermentor cultivation of *G. reessii*. (a) Cell [dry weight], MBA, and HMB concentrations and molar HMB yield; (b) the specific production rate (qP) of HMB. The arrow indicates the start of MBA addition and the switching of pH from 6.5 to 7.0.

HMB production step. It was also noted that the production rate (qP) was as high as 0.40 g of HMB/g of cells [dry weight]/h after the addition of MBA. The specific production rate of *G. reessii* was maintained at a high level until 118 h, after which it decreased.

DISCUSSION

Precise details of the pathway involved in the conversion of MBA to HMB are not yet known. However, it is likely that conversion of MBA to HMB follows a β oxidation pathway. We believe that MBA is first converted into MBA-coenzyme A (CoA) by an acyl-CoA synthetase (EC 6.2.1.3), followed by conversion to methylcrotonyl-CoA by acyl-CoA dehydrogenase (EC 1.3.99.10). Subsequent hydration to HMB-CoA by an enoyl-CoA hydratase (EC 4.2.1.17) and hydrolysis would give HMB.

In preliminary experiments, three strains known to contain β oxidation capabilities were tested for their abilities to transform MBA into HMB. These were *C. rugosa* ATCC 10570 (2, 5), *Rhodotorula rubra* IFO 889 (4), and *Galactomyces reessii* CBS 179.60 (3). Since *C. rugosa* converts isobutyric acid to β-hydroxyisobutyric acid, it was expected to convert MBA to HMB. However, this organism rapidly consumed MBA, yielding a mixture of unknown metabolites but not HMB. *R. rubra*, which catalyzes the conversion of ferulic acid to vanillic acid by β oxidation, was unable to utilize MBA. Of the cultures examined, *G. reessii* was able to convert MBA to HMB, albeit relatively inefficiently.

Conversions of MBA to HMB by *G. reessii* were sensitive to different relative volumes of culture in 125-ml flasks (Fig. 1 and Table 1). This result suggested that the conversion of MBA to HMB was sensitive to DO, although other factors may be involved. In addition, glucose as a carbon and energy source was also required to increase HMB yields and to prevent its consumption and degradation by the organism (Table 1). It is likely that glucose, through catabolite repression, diminishes the utilization of HMB as a source of carbon and energy. Thus, in a jar fermentation, DO was maintained at 20% air saturation throughout the incubation, and glucose feed solution was supplied continuously. Cells grew more efficiently at slightly acidic pH, while HMB was degraded much faster under acidic condition (Fig. 2b). This may be due to the more ready transport of protonated HMB into cells and its assimilation for cell growth at acidic pH. Thus, in the two-step, fed-batch fermentation, the culture was controlled at pH 6.5 during cell growth and at pH 7.0 during HMB production. Since MBA inhibits HMB production, it was crucial to maintain substrate concentrations of less than 20 g/liter.

In this study, an HMB titer of 38 g/liter was obtained in 0.5 M conversion yield by a two-step, fed-batch fermentation of a *G. reessii* feeding system by controlling the additions of both MBA and glucose. The fermentation process described here gives good yields of HMB with the use of an inexpensive starting material under mild reaction conditions. This work demonstrates the feasibility of producing HMB by the biocatalytic transformation of MBA.

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