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Ammonia Production from Yeast Extract and Its Effect on Growth of the Hyperthermophilic Archaeon *Sulfolobus solfataricus*

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Utilization of yeast extract and formation of byproduct metabolite were investigated for hyperthermophilic archaeon *Sulfolobus solfataricus* (DSM 1617). In both batch and fed-batch cultivations of *S. solfataricus*, maximal cell density, NH_4^+ ion production and pH change were highly dependent on the ratio of yeast extract to glucose in the medium. Variation of NH_4^+ ion level was identified as a major cause of pH change during cultivation, and acidification of culture broth was attributed to consumption of NH_4^+ ions rather than formation of acid byproducts. It was also observed that increase of NH_4^+ ion concentrations in the medium resulted in greater degree of growth inhibition.

Key words: hyperthermophile, archaea, *Sulfolobus solfataricus*, ammonia, yeast extract, fed-batch

Recent isolation of hyperthermophilic archaea greatly expands the scope of biotechnology and opens a way to operate biotechnology processes up to or over the boiling point of water [1, 2]. However, there are many difficulties in the application of hyperthermophiles due to their unknown characteristics of growth and poor growth yield [3, 4]. To obtain dense culture of hyperthermophiles, therefore, more informations on their physiological characteristics such as utilization of nutrients and metabolite production are needed [5, 6].

Yeast extract has been widely used as an essential nutrient for growth of many archaea. Most of the acidophilic hyperthermophiles, which are archaea of the orders *Sulfolobales* and *Thermoplasmales*, also require yeast extract for their growth and maintenance, and interestingly many of them exhibit growth inhibition at higher yeast extract concentrations [7-11]. However, little is known about the utilization of yeast extract in hyperthermophiles despite its importance in cultivation of hyperthermophiles.

In the present work we studied the utilization pattern of yeast extract in *Sulfolobus solfataricus*, a most well-known hyperthermophilic acidophile. We measured pH and NH_4^+ ion concentrations along with cell densities during fed-batch cultivation of *S. solfataricus*. The effects of yeast extract on cell growth were examined by varying the ratio of yeast extract to glucose in the fed medium. These studies demonstrate that NH_4^+ ion can be produced or consumed depending on the amount of yeast extract in the medium and that the change in NH_4^+ ion level is a major cause of pH variation and growth inhibition during cultivation of *S. solfataricus*.

S. solfataricus (DSM 1617), which was isolated from volcanic hot spring in Italy, was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Batch cultivations were carried out at 78℃ in 500 mL screw-cap flasks with a working volume of 50 mL. GYM medium [11], which was composed of glucose (G) 3.0 g, yeast extract (Y) 3.0 g, and modified Allen's basal salt (M) in 1 liter of distilled water, was used as a base medium for batch cultivation. Modified Allen's basal salt contains $(NH_4)_2SO_4$ 1.3 g, KH_2PO_4 0.28 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, $CaCl_2 \cdot 2H_2O$ 70 mg, $FeCl_3 \cdot 6H_2O$ 20 mg, $Na_2B_4O_7 \cdot 10H_2O$ 4.5 mg, $MnCl_2 \cdot 4H_2O$ 1.8 mg, $ZnSO_4 \cdot 7H_2O$ 0.05 mg, $CuCl_2 \cdot 2H_2O$ 0.05 mg, $VOSO_4 \cdot 5H_2O$ 0.04 mg, $Na_2MoO_4 \cdot 5H_2O$ 0.03 mg, and $CoSO_4 \cdot 7H_2O$ 0.01 mg per liter [7]. Yeast extract was purchased from Difco (USA) and all other reagents used were analytical grade and obtained from Sigma (USA).

Fed-batch cultivations were carried out in a bench-top fermentor with a working volume of 2.3 l (KLF 2000, Bioengineering AG, Switzerland). Culture temperature and aeration rate were 78℃ and 1 vvm, respectively. Cells grown in GYM medium were used as an inoculum. After cultivating the cells in GYM medium for 45-50 h, feed medium was supplied and fed-batch operation started. Feed medium used in fed-batch cultures was composed of glucose and yeast extract (pH 3.0). A constant-volume fed-batch protocol [12] was applied to compensate water evaporation during fed-batch operations.

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Feed rate was controlled to maintain the residual glucose concentration at around 3 g/l, an optimal residual glucose concentration for the growth of *S. solfataricus* [11].

Cell density was determined by turbidity measurements at 540 nm and correlated to dry weight. For the determination of dry cell weight, cells were washed twice with distilled water, and dried for 48 h at 110℃. Residual glucose concentration was determined using *o*-toluidine reagent kit (Sigma, USA). Concentration of ammonia was measured by the phenate method [13]. Organic acids in the culture broth were analyzed by HPLC (Knauer, Germany) and a UV detector (210 nm) with a Spherisorb Octyl column (Supelco, USA).

In order to investigate the effect of yeast extract on cell growth, batch cultures were carried out by adding different amount of yeast extract to the culture medium (Table 1). Growth of *S. solfataricus* was enhanced with the addition of yeast extract. When yeast extract was not included in culture medium (GM medium), maximal cell density was reduced to 46% of that obtained in GYM medium (Table 1(A)). Although cell growth was promoted by the addition of 3 g/l yeast extract to GM medium (GYM medium), further addition of yeast extract to GYM medium resulted in growth inhibition (Table 1(B)). During this experiment we noticed significant differences in pH depending on the content of yeast extract in the medium. The final pH of culture broth increased in the presence of yeast extract, whereas culture pH decreased when yeast extract was not included in the medium (data not shown).

Fed-batch operation is frequently used for high cell density culture and, if cell growth is inhibited by excess nutrients, this mode of operation is very useful because the level of nutrients in the fermentor can be maintained at a low level. Considering the flask culture results, it seemed likely that there might be more drastic change of culture pH and formation of byproduct metabolites in fed-batch operation due to continuous feeding of yeast extract. In order to examine the effect of yeast extract on cell growth and on pH variation, fed-batch operations were conducted in a bench-top fermentor with four different ratios of yeast extract to glucose (Y/G) in the feed medium (0, 0.2, 1, 3). An increase of the Y/G ratio in feeding solution means a corresponding increase of total amount of yeast extract fed into the culture broth.

Table 1. Effect of yeast extract and ammonium sulfate on the growth of *S. solfataricus*.

	Culture medium	Relative cell density[a] (%)
(A)	GM	46
	GYM	100
(B)	GYM + 1 g/l yeast extract	94
	GYM + 3 g/l yeast extract	68
	GYM + 5 g/l yeast extract	49
(C)	GYM + 25 mM $(NH_4)_2SO_4$	92
	GYM + 50 mM $(NH_4)_2SO_4$	76
	GYM + 100 mM $(NH_4)_2SO_4$	65

[a]Cells were cultivated in screw-cap flasks for 90 h and cell densities were measured as described in the text. Cell densities obtained in specified culture medium were normalized to that obtained in GYM.

As can be seen from Fig. 1A, the highest cell density was obtained at the Y/G ratio of 0.2, and the maximum cell density under this condition was 5.3 g/l. Cell growth was inhibited at higher yeast extract concentrations; at the Y/G ratio of 3, for example, maximum cell density was only 1.2 g/l. When the feed medium that lacked yeast extract was used, cell growth was markedly retarded by introduction of feed medium to the fermentor. Interestingly, there were two distinct patterns of pH variation depending on Y/G ratios (Fig. 1B). At high Y/G ratios (≥ 1) the pH of culture broth increased from 3.0 to 5.9, whereas at low Y/G ratios (≤ 0.2) it decreased below 2.0. Analysis of culture broth revealed that residual NH_4^+ ions increased in the

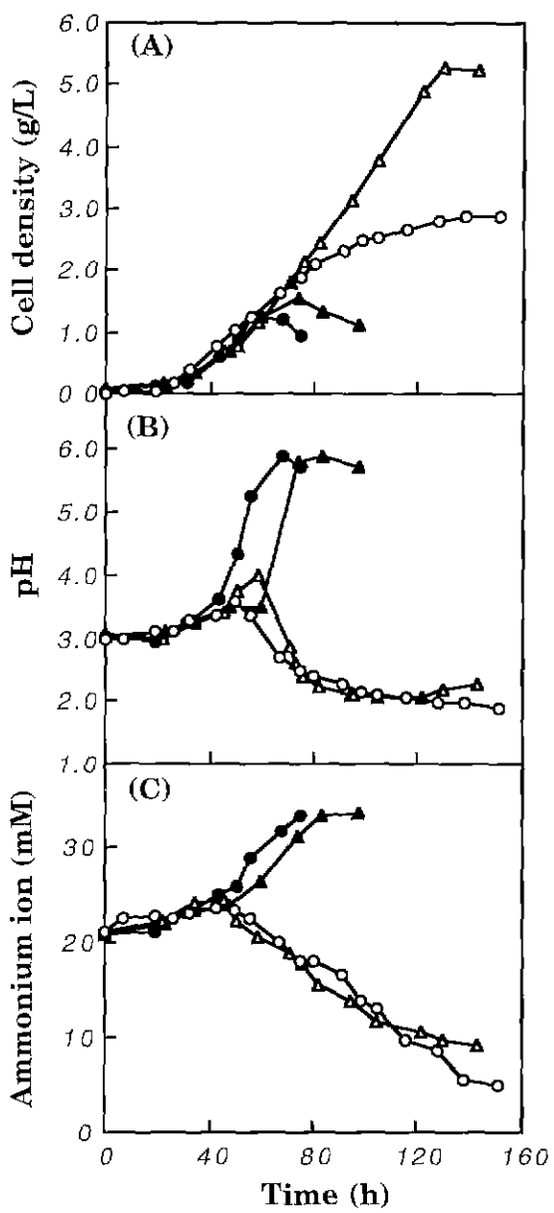

Fig. 1. Time profiles of cell growth (A), pH (B), and NH_4^+ ions (C) in fed-batch cultures without pH control. The ratio of yeast extract to glucose in the feed medium was 0 (○), 0.2 (△), 1 (▲), or 3 (●).

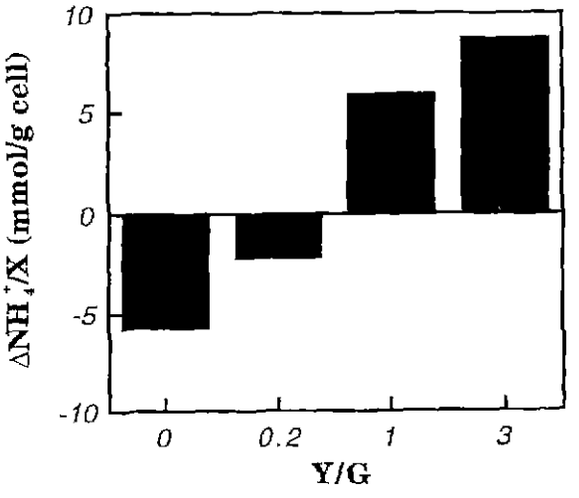

Fig. 2. Dependence of NH_4^+ production (or consumption) on the ratio of yeast extract to glucose in the feed medium (Y/G). ΔNH_4^+ and X represent the change of NH_4^+ ion concentration due to cell growth and the maximum cell density, respectively.

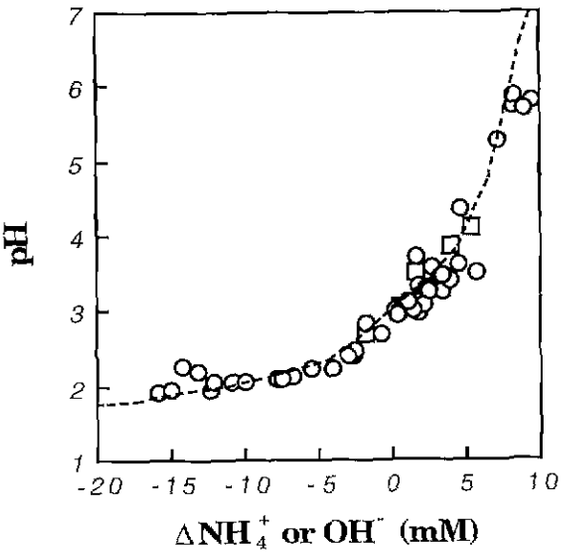

Fig. 3. Effect of NH_4^+ ions on pH change of culture medium. Changes of NH_4^+ ions in flask cultures (□) and fed-batch cultures (○) are plotted against the final pH of culture broth. Titration curve of GYM medium (dashed line) is also shown for comparison.

former cases ($Y/G \geq 1$) whereas NH_4^+ levels were reduced in the latter cases ($Y/G \leq 0.2$) (Fig. 1C).

The consumption of NH_4^+ ions by cells becomes more apparent when the specific changes of NH_4^+ ions ($\Delta NH_4^+/X$) are plotted against the Y/G ratio (Fig. 2). The value of $\Delta NH_4^+/X$ changes from negative to positive with increasing Y/G ratio, which indicates that NH_4^+ ions are produced as a metabolite from yeast extract at high Y/G ratios (≥ 1) while NH_4^+ ions are consumed as a nitrogen source at low Y/G ratios (≤ 0.2).

The pH increase at higher Y/G ratios can be explained by the production of ammonia from yeast extract. Explanation for the pH decrease at low Y/G ratios is rather complicated, since reduction of culture pH can be caused by either production of acid byproducts or consumption of base metabolites. To examine the possibility of organic acid production, culture broth was analyzed with a HPLC system. However, no appreciable peak that corresponded to acetate, propionate, lactate, citrate or succinate was detected in the chromatograms. This result suggests that the pH decrease at low Y/G ratios is attributed to consumption of NH_4^+ ions rather than formation of acid byproducts.

To examine whether the pH variation during cultivation is solely governed by the NH_4^+ ion changes in the culture broth, GYM medium was titrated with HCl (1.0 N) or NH_4OH (1.1 N) and then the pH versus NH_4^+ ion changes in previous cultures were compared with a titration curve. Since ammonia is ionized in an aqueous solution

$$NH_3 + H_2O = NH_4^+ + OH^-$$

accumulation or reduction of NH_4^+ ions will result in an equivalent increase or decrease of OH^- ions in the culture broth.

As shown in Fig. 3, the pH versus ΔNH_4^+ data obtained in batch and fed-batch cultures coincided well with the titration curve of GYM medium. Based on this result, we concluded that the pH increase at high yeast extract contents was ascribed by the generation of NH_4^+ ions and that the pH decrease at low yeast extract contents resulted from the consumption of NH_4^+ ions.

Finally, the effect of NH_4^+ ions on cell growth was examined in flask cultures. As shown in Table 1(C), extra addition of ammonium sulfate to GYM medium resulted in growth inhibition of *S. solfataricus*. When 100 mM ammonium sulfate was added to GYM medium, for example, cell density was reduced to about two thirds of that obtained in GYM medium. However, the inhibitory effect of ammonium sulfate was less profound than that of yeast extract. This may indicate that other unknown factors than NH_4^+ ion accumulation contribute to growth inhibition at higher yeast extract concentrations. This possibility is currently under investigation in our laboratory.

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