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Cultivation of the Hyperthermophilic Archaeon *Sulfolobus solfataricus* in Low-Salt Media

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Two low-salt complex media, bactopeptone and desalted yeast extract, were used for high density cultivation of the hyperthermophilic archaeon *Sulfolobus solfataricus* (DSM 1617). Bactopeptone, which has low mineral ion content among various complex media, was good for cell growth in batch cultures; the maximal cell density in bactopeptone was comparable to that in yeast extract. However, cell growth was rather poor when bactopeptone was added by the fed-batch procedure. Since several vitamins are deficient in bactopeptone, the effect of vitamins on cell growth was examined. Among the vitamins tested, pyridoxine was found to improve the growth rate of *S. solfataricus*. To reduce the growth inhibition caused by mineral ions, yeast extract was dialyzed against distilled water and then fed-batch cultures were carried out using a feed medium containing desalted yeast extract. Although the concentrations of mineral ions in yeast extract were significantly lowered by the dialysis procedure, fed-batch cultivation with desalted yeast extract was unsatisfactory. To examine whether low molecular weight solutes in yeast extract are crucial for cell growth, we investigated the effect of trehalose, a most abundant compatible solute in yeast extract, on the growth pattern. Cell densities were increased and the length of the lag phase was markedly shortened by the presence of trehalose, indicating that trehalose plays an important role in the growth of *S. solfataricus*.

Key words: hyperthermophile, archaea, *Sulfolobus solfataricus*, low-salt nutrient, bactopeptone, yeast extract, vitamin, trehalose

INTRODUCTION

Living cells have long been known to make their lives under very narrow range of temperatures. Recent isolations of microorganisms capable to grow even in boiling water, however, have made the traditional view on optimal range of growth temperature for living organisms to modify [1-3]. The "highly heat-loving" hyperthermophiles, most of which found in volcanic areas and deep-sea hydrothermal vents, have attracted many interests from biotechnological aspect due to their potential prospects in industrial applications as well as from academic curiosities about how cell components stabilize against heat [4-7].

Industrial processes utilizing the hyperthermophiles are expected to be quite useful for technical and environmental purposes. Biocatalysts (whole cells or enzymes) obtained from the hyperthermophiles have been reported to be stable at around 100℃ and in organic solvents. Thermostable and robust characteristics of hyperthermophiles can therefore be applied to the biocatalysis under extreme environments. Despite high potentials of hyperthermophiles, however, their applications have been quite limited due to poor growth in a large scale fermentor, which is mainly ascribed to little information on cell physiology of hyperthermophiles and the lack of cell culture techniques at high temperatures [8,9].

Recently, we have studied high cell density cultivation of *Sulfolobus solfataricus*, a hyperthermophilic archaeon with maximum growth temperature of around 90℃. According to our previous studies [10-13], the growth of *S. solfataricus* was markedly promoted by the addition of yeast extract to the culture medium [10, 12]. However, the use of yeast extract as a feed medium for fed-batch operation was hampered due to inhibition of cell growth at high yeast extract concentrations. Main reason for the growth inhibition by excess yeast extract was found to be attributed to accumulation of mineral ions in the culture broth due to continuous feeding of yeast extract and the inhibitory effect of mineral ions on the growth of *S. solfataricus* [13].

In the present work we tested two kinds of low-salt media for high cell density cultivation of *S. solfataricus*. Bactopeptone, a complex nutrient which contains the lowest level of mineral ions among various peptones, and desalted yeast extract, which can be prepared by dialyzing yeast extract, were chosen as culture media and the growth of *S. solfataricus* in these low-salt media was investigated in batch and fed-batch cultivations.

MATERIALS AND METHODS

Strain and Media

S. solfataricus (DSM 1617), which was isolated from volcanic hot spring in Italy, was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Base medium used for

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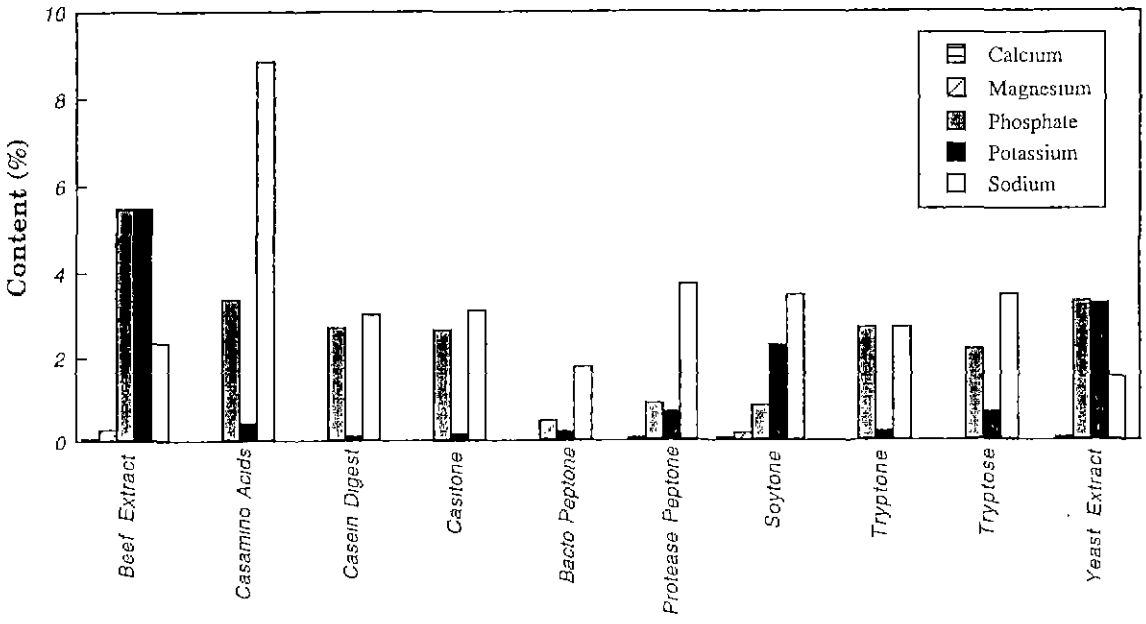

Fig. 1. Content of major mineral ions in various complex media. The data provided by the supplier (Difco) were used without further verification.

liquid cultivation in fermentor was composed of glucose 3.0 g, complex nutrient (yeast extract or bactopeptone) 3.0 g, and modified Allen's basal salt medium in 1 liter of distilled water (pH 3.0). The modified Allen's basal salt medium contains $(NH_4)_2SO_4$ 1.3 g; KH_2PO_4 0.28 g; $MgSO_4 \cdot H_2O$ 0.25 g; $CaCl_2 \cdot H_2O$ 70 mg; $FeCl_3 \cdot H_2O$ 20 mg; $Na_2B_4O_7 \cdot H_2O$ 4.5 mg; $MnCl_2 \cdot H_2O$ 1.8 mg; $ZnSO_4 \cdot H_2O$ 0.05 mg; $CuCl_2 \cdot H_2O$ 0.05 mg; $VOSO_4 \cdot H_2O$ 0.04 mg; $Na_2MoO_4 \cdot H_2O$ 0.03 mg; $CoSO_4 \cdot H_2O$ 0.01 mg per liter [14]. For the test of trehalose effect on growth of *S. solfataricus*, GM medium composed of glucose 3.0 g/L and modified Allen's basal salts was used. GGM medium which was used for the test of vitamin effect on cell growth was prepared by supplementing L-glutamate (3.0 g/L) to GM medium. Yeast extract and bactopeptone were purchased from Difco and all other reagents used were analytical grade and obtained from Sigma.

Cultivation of the Microorganism

Cells were cultivated as described in our previous works [11-13]. Cultivation in a screw-cap flask (working volume; 50 mL) was carried out at 78℃ in a shaking water bath with agitation speed of 100 rpm. Cultivation in a jar fermentor was carried out at 78℃ in a bench-top fermentor system with a working volume of 2.3 L (KLF2000, Bioengineering AG, Switzerland). Cells grown in screw-cap flasks at 78℃ in a shaking water bath were used as an inoculum. In fed-batch experiments, the feed medium was supplied after cultivating the cells for 45~50 hr and fed-batch operation started. Feeding rate was adjusted periodically to maintain the residual glucose concentration between 2 and 4 g/L. The pH of culture broth was automatically controlled at 3.0 by the addition of H_2SO_4 (3.8 N) or NaOH (2.0 N), and the DO level was kept above 30% of air saturation by changing the agitation speed.

Analyses

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 hr at 110℃. Residual glucose concentration was determined using *o*-toluidine reagent kit (Sigma). The concentration of ammonium ion was measured by the phenate method [15]. Mineral ions such as potassium, sodium, calcium and magnesium were analyzed with an atomic emission spectrophotometer (Plasma-Therm Model HFP-2500, Baird, U.S.A.). Protein content in desalted yeast extract was analyzed with the Bradford method.

RESULTS AND DISCUSSION

Use of Bactopeptone

As shown in Fig. 1, bactopeptone (BP) contains the lowest level of mineral ions (calcium, magnesium, phosphate, potassium and sodium) among various complex nutrients that are widely used as culture media. For instance, the content of K^+ ion in bactopeptone was about 30 times lower than that in yeast extract. For this reason bactopeptone was selected as a substitute for yeast extract and used for the cultivation of *S. solfataricus*.

Effect of bactopeptone on growth of *S. solfataricus* was examined in both batch and fed-batch cultivations. In batch cultures, the growth of *S. solfataricus* in a medium containing bactopeptone was similar to that observed in a medium containing yeast extract (Fig. 2a). In the case of fed-batch cultures, however, cell growth was found to be quite poor compared to that obtained with yeast extract (Fig. 2b). The low growth rate in fed-batch culture was not improved by increasing the ratio of bactopeptone to glucose (BP/G) in the feed medium.

It was observed that glucose and NH_4^+ ions were not depleted during fed-batch cultivations with bactopeptone. Also, there was no significant accumulation of mineral ions when the cells were cultivated in a bactopeptone-based medium. In view of these observations,

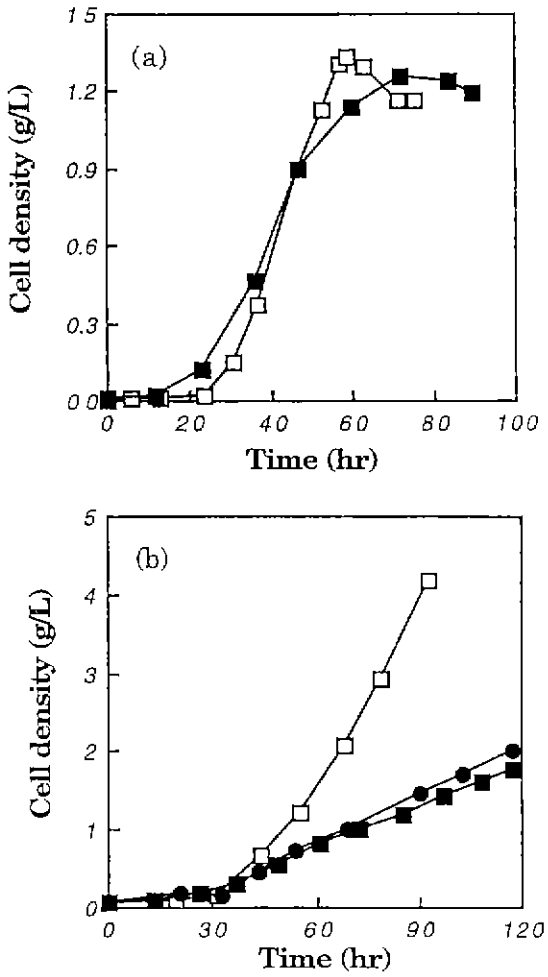

Fig. 2. Growth of *S. sofataricus* on bactopeptone (BP) or yeast extract (YE) in batch (a) and fed-batch (b) cultures. Symbols in (a): (□), batch growth on YE; (■), batch growth on BP. Symbols in (b): (□), fed-batch growth on YE (YE/G ratio = 1/4); (■), fed-batch growth on BP (BP/G ratio = 1/4); (●), fed-batch growth on BP (BP/G ratio = 1).

poor growth on bactopeptone was regarded to be attributable to a lower vitamin content. Vitamins are organic compounds required in small amounts for growth and function of organism and form parts of prosthetic groups or active centers of enzymes, playing essential roles in cell metabolism. Taking into account the roles of vitamins, it is likely that vitamins are indispensible for dense growth of *S. solfataricus*. According to the data provided by the supplier (Difco), there are several vitamins the contents of which are quite low in bactopeptone. For instance, the amount of pyridoxine in bactopeptone is about 25-fold lower than that in yeast extract. We therefore investigated the effect of these vitamins on growth of *S. solfataricus* in the next.

Vitamins which exist in a lower level in bactopeptone than in yeast extract include p-aminobenzoic acid, d-biotin, folic acid, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, and thiamine. Each vitamin or mixture of eight vitamins was added to a defined medium (GGM) composed of glucose 3 g/L, L-glutamate 3 g/L, and modified Allen's basal salts. The amount of each vitamin added to bactopeptone was determined by referring its corresponding amount in yeast extract; 2289.0 mg/L (p-aminobenzoic acid), 9.9 mg/L (d-biotin), 4.5 mg/L (folic acid), 1793.7 mg/L (nicotinic acid), 819.9 mg/L (pantothenic acid), 129.6 mg/L (pyridoxine), 349.5 mg/L (riboflavin), and 1589.7 mg/L (thiamine).

Table 1. Effect of vitamins on growth of *S. solfataricus* in batch cultures[a]

Vitamins	Growth rate (%)	Cell density (%)	Lag time (%)
mixture of vitamins	138	103	73
p-aminobenzoic acid	98	99	95
d-biotin	96	98	107
folic acid	108	101	102
nicotinic acid	94	97	102
panthothenic acid	101	100	99
pyridoxine	140	101	75
riboflavin	108	97	101
thiamine	106	99	105

[a] The values of growth rates, cell densities, and lag time were normalized to those obtained in the medium without vitamin.

Effects of supplemented vitamins on the growth of *S. solfataricus* (growth rate, maximum cell density, and lag time) are shown in Table 1. Growth rate was enhanced when mixture of eight vitamins was added to GGM medium. In order to identify the vitamin which enhanced the growth of *S. solfatraicus*, the effect of each vitamin on cell growth was also examined. As can be seen from Table 1, pyridoxine was found to improve the growth rate. It has been reported that *S. solfataricus* has different composition of vitamins from other mesophilic microorganisms and, interestingly, the levels of pyridoxine and riboflavin in *S. solfataricus* are quite higher than those in other bacteria and eucaryotic microorganisms [16]. This may be associated with the significant enhancement of growth rate by pyridoxine.

Use of Desalted Yeast Extract

Since bactopeptone was not appropriate for high cell density cultivation of *S. solfataricus*, we attempted to use another low-salt medium, desalted yeast extract (dYE), as an alternative. For the preparation of dYE, yeast extract solution was dialyzed against distilled water using a cellulose membrane (Spectra/Por1, Spectrum Co., U.S.A.).

By the pretreatment of yeast extract, the content of mineral ions was reduced to less than one tenth of the initial value within 12 hr (Fig. 3a) while a half of proteins was still retained after dialysis (Fig. 3b).

With the use of dYE, *S. solfataricus* grew well up to 110 hr in a fed-batch cultivation, but thereafter the growth rate was dropped (Fig. 4, I-a). When the residual NH_4^+ ion concentration was analyzed, there was an exact coincidence between the drop of growth rate and the depletion of residual NH_4^+ ion in culture broth (Fig. 4, II-a). When NaOH solution used for pH titration was replaced with NH_4OH, cell densities were further increased. This implies that depletion of nitrogen sources (NH_4^+) can be a reason for the cessation of cell growth in the previous fed-batch experiments with dYE (Fig. 4, I-b, II-b).

Since residual NH_4^+ ions were depleted even the case that pH was titrated with NH_4OH, ammonium sulfate was supplied intermittantly into the culture broth to prevent the shortage of nitrogen sources. Contrary to our expectation, however, no further growth improvement was observed even though the NH_4^+ ion concen-

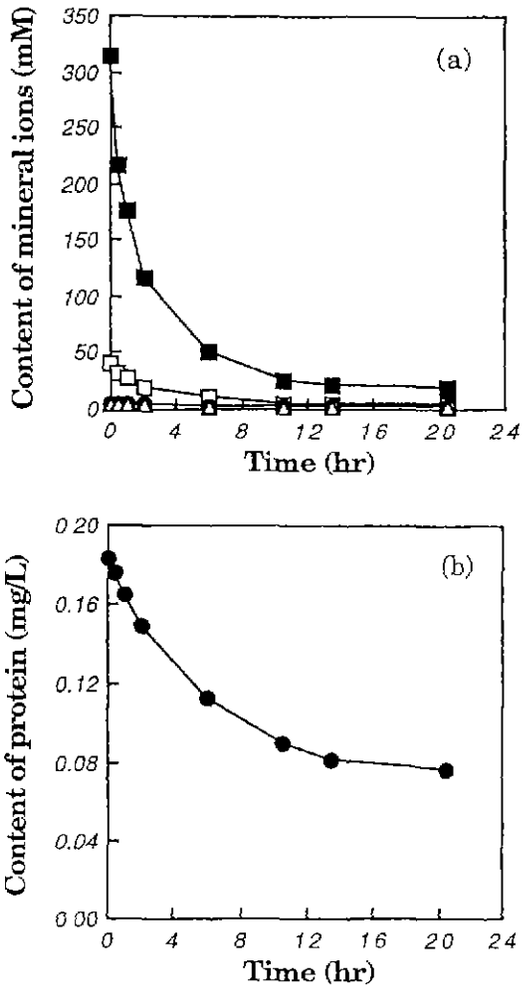

Fig. 3. Changes in the concentration of mineral ions (a) and proteins (b) during dialysis of yeast extract using a cellulose membrane (molecular weight cutoff, 6,000~8,000). Symbols in (a): (■), K^+; (□), Na^+; (○), Ca^{2+}; (△), Mg^{2+}.

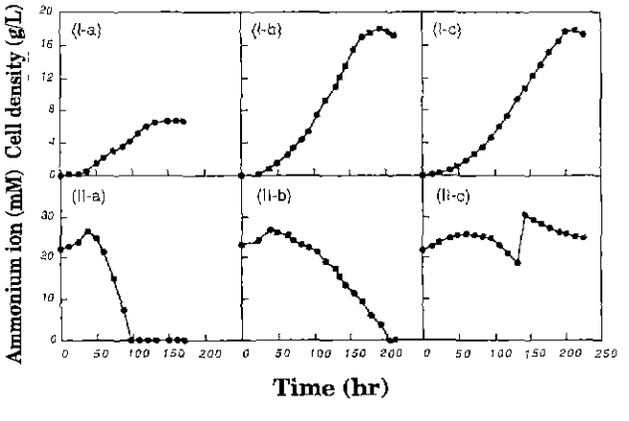

Fig. 4. Time course profiles of cell growth (I) and residual NH_4^+ ion concentration (II) in fed-batch cultivation of *S. sofataricus* using a feed medium composed of desalted yeast extract and glucose (dYE/G ratio = 1/3). (a) pH titration with NaOH; (b) pH titration with NH_4OH; (c) pH titration with NH_4OH and intermittant addition of $(NH_4)_2SO_4$.

tration in the broth remained high (Fig. 4, I-c, II-c).

The fed-batch experiments with dYE indicate that yeast extract contains some other factors that are important for the growth of *S. solfataricus*. We focussed on finding low molecular weight compounds (LMWCs) which might play an important role in the growth and thermoadaption of *S. solfataricus* because the concentration of LMWCs should have been reduced along with mineral ions during preparation of dYE using a dialysis membrane.

Table 2. Effect of trehalose on growth of *S. solfataricus* in batch cultures[a]

Trehalose (g/L)	Growth rate (%)	Cell density (%)	Lag time (%)
0.1	117	112	48
0.5	141	109	47
1.0	142	114	47
3.0	158	112	36

[a] The values of growth rates, cell densities, and lag time were normalized to those obtained in the medium without trehalose.

Vitamins can be classified as LMWCs essential for the growth of *S. solfataricus* and their effect on cell growth are shown in Table 1. Another LMWCs required for efficient growth and thermoadaption of *S. solfataricus* may be compatible solutes, which are defined as small organic solutes accumulated in organisms against osmotic stress, drying, or heat shock. According to recent reports, various kinds of compatible solutes are accumulated intracellularly in the process of thermoadaptation of hyperthermophiles as well as in their osmoadaptation [17,18].

Trehalose, one of the most well-known compatible solutes, has been reported to accumulate to high levels (up to 20% of the dry weight) when yeast cells are in the stationary phase or exposed to high temperatures [19-21]. In view of these reports, it is most likely that trehalose contained in yeast extract is essential for the thermoadaptation of *S. solfataricus*. To explore this possibility, we examined the effect of trehalose on growth of *S. solfataricus*. In this experiment, different amount of trehalose was added to GM medium and the growth of *S. solfataricus* was monitored. As shown in Table 2, growth rate was enhanced with the addition of trehalose and, especially, the length of the lag phase of *S. solfataricus* was significantly shortened by the presence of trehalose. This indicates that exogenously supplied trehalose can also help the adaptation of *S. solfataricus* to high temperature environment.

In this work, we examined the growth of *S. solfataricus* in low-salt media in order to reduce the growth inhibition caused by mineral ions. During this investigation we found that, in addition to low concentration of mineral ions, the presence of LMWCs (e.g., vitamins and compatible solutes) in the culture medium is required for efficient cell growth. Considering the results presented in this work, it appears that compatible solutes play an important role in the growth of hyperthermophiles. We are currently investigating the effect of various compatible solutes on the growth of hyperthermophiles and the results will be reported in the near future.

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