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Studies on nutritional and oxygen requirements for production of L-asparaginase by *Enterobacter aerogenes*

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Abstract The carbon and nitrogen sources most suitable for L-asparaginase production by *Enterobacter aerogenes* were selected and their concentrations optimized in shake-flask cultures. Sodium citrate (1.0%) and diammonium hydrogen phosphate (0.16%) proved to be the best sources of carbon and nitrogen, respectively. Nitrogen catabolite repression of enzyme formation was absent in this bacterium. Cultivation in a reactor showed that the dissolved oxygen level is the limiting factor for L-asparaginase production by *E. aerogenes*. Glucose was found to be a repressor of enzyme synthesis. Asparagine was absent intracellularly when the L-asparaginase level was high. An increase in the extracellular alanine level when the dissolved oxygen remained low indicated a shift from aerobic to fermentative metabolism.

Introduction

L-Asparaginase is widely used in the chemotherapy of acute lymphoblastic leukemia. Toxic side effects of the currently used clinical preparations have necessitated the search for alternative microbial sources. Some recent reports are *Tetrahymena pyriformis* (Triantafillou et al. 1988), *Corynebacterium glutamicum* (Mesas et al. 1990), *Cylindrocarpon obtusisporum* (Raha et al. 1990), *Pseudomonas stutzeri* (Manna et al. 1995), *Candida utilis* (Kil et al. 1995) and *Rhodosporodium toruloides* (Ramakrishnan and Joseph 1996). Compared to the methods involving five or six steps described earlier, we reported a simple downstream process designed to isolate the intracellular L-asparaginase from *Enterobacter aerogenes* (Mukherjee et al. 1999a). In this communication, we report the optimization of the nutrients and process conditions.

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Materials and methods

Organism

E. aerogenes strain NCIM 2340 was obtained from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. It was grown on nutrient agar slants for 24 h at 37 °C, maintained at 0–2 °C and subcultured every month.

Cultivation in shake flasks

Effect of different carbon sources and nitrogen sources on the production of L-asparaginase by E. aerogenes

The effects of carbon sources on cell growth and L-asparaginase production were studied in the basal semisynthetic medium consisting of $NaNO_3$, 0.2%; $MgSO_4 \cdot 7H_2O$, 0.05%; $FeSO_4 \cdot 7H_2O$, 0.001%; KCl, 0.05%; K_2HPO_4, 0.1% and yeast extract, 0.05% (pH 7.0). The carbon sources were studied at a concentration of 1.0% in the basal medium. 0.1 ml of a 24-h cell suspension was used to inoculate each 250-ml fermentation flask containing 50 ml medium. The flasks were shaken at 37 °C, samples being taken at 18, 24 and 36 h for determination of cell growth and enzyme potency.

The medium for testing different simple and complex nitrogen sources contained the basal medium after omission of $NaNO_3$ while sodium citrate at 1.0% concentration was used as the carbon source.

Effect of glucose

A study was made to determine the effect of addition of glucose at different levels and times on enzyme production, cell growth and pH of the medium. The medium contained basal mineral salts plus diammonium hydrogen phosphate (0.16%) and glucose or sodium citrate, singly or in combination as shown in Table 1. The precultures for all the cultivations were grown in the basal medium with sodium citrate (1.0%).

Table 1 Effect of glucose on cell growth, pH changes of the broth and L-asparaginase production by *Enterobacter aerogenes* NCIM 2340

Carbon source	Enzyme activity (IU/ml)		Cell concentration (g/l)		pH		
	18 h	24 h	18 h	24 h	12 h	18 h	24 h
Sodium citrate (1.0%)	0.43 ± 0.10	0.60 ± 0.02	2.20 ± 0.10	2.50 ± 0.05	8.25	8.85	9.20
Glucose (1.0%)	0.36 ± 0.01	0.23 ± 0.02	2.45 ± 0.05	2.14 ± 0.10	5.90	6.70	6.00
Glucose (0.5%) + sodium citrate (0.5%)	0.57 ± 0.02	0.32 ± 0.01	2.68 ± 0.10	2.44 ± 0.05	7.50	8.50	8.54
Glucose (0.5%) at 0 h + glucose (0.5%) at 12 h	0.43 ± 0.02	0.40 ± 0.02	2.08 ± 0.15	2.55 ± 0.07	6.70	7.00	6.40
Sodium citrate (0.5%) at 0 h + glucose (0.5%) at 12 h	0.49 ± 0.10	0.54 ± 0.02	1.96 ± 0.05	2.83 ± 0.05	7.90	8.37	8.50

Cultivation in a 2.0-l bioreactor

The medium

1.5 l medium, developed after optimization of carbon, nitrogen, mineral salts and trace element requirements, consisted of trisodium citrate, 0.75% $(NH_4)_2$ HPO_4, 0.2%; $MgSO_4 \cdot 7H_2O$, 0.02%; $FeSO_4 \cdot 7H_2O$, 0.002%; K_2HPO_4, 0.0125%; yeast extract, 0.15%, silcone oil (0.1% as antifoam). Preculture for the cultivation with glucose was grown in the basal medium with sodium citrate (1.0%).

The process and on-line data

3.0 ml of a 24-h shake-flask culture was used to inoculate 1.5 l medium contained in a 2.0-l bioreactor fabricated at the mechanical workshop of the Institut für Technische Chemie der Universität Hannover, Hannover, Germany which was attached to a Biostat M process unit (Braun Biotech International, Melsungen, Germany). The process was carried out at a controlled pH (8.0) and temperature (37 °C) which were found to be optimal. All on-line data were acquired with the Micro MFCS software program from Braun Biotech International, Melsungen, Germany.

Effect of aeration rate and agitation

The effects of aeration and agitation were investigated under four different process conditions; (a) aeration rate 1.0 vvm, agitation 400 rpm; (b) aeration rate 1.0 vvm, agitation 700 rpm; (c) aeration rate 1.5 vvm, agitation 700 rpm; (d) aeration rate 1.5 vvm, agitation 500 rpm for 6 h followed by an aeration rate of 0.5 vvm, agitation 200 rpm for 10 h.

Off-line analyses

L-Asparaginase activity

The cells were separated from the medium by centrifugation (10,000 rpm) at 5 °C and were shaken vigorously with 20 ml phosphate buffer (pH 7.0) containing Triton X-100 (0.01%) for 5 min. After centrifugation, the cells were suspended in 1.5 ml sodium borate buffer pH 8.65, and assayed for L-asparaginase activity. The reaction mixture containing 1.0 ml sodium borate buffer pH 8.65, 100 µl cell suspension and 1.0 ml 0.04 M L-asparagine was incubated at 37 °C for 10 min. The reaction was stopped by the addition of trichloroacetic acid. The mixture was centrifuged and the ammonia released in the supernatant was determined by the DIN 38406 test (DIN 38406, Bestimmung des Ammonium Stickstoffs; Okt., 1983). One unit of L-asparaginase is defined as the amount of enzyme which liberates 1.0 µmol ammonia in 1 min at 37 °C.

Total cellular protein

Samples of 1.5 ml cell suspension in borate buffer were diluted 10 times (except for the samples after 2 h and 4 h) and the protein determined by the Bio Rad test kit (Source: Bio-Rad Laboratories, Hercules, California, USA).

Citrate

The supernatant obtained after separating the cells was diluted 10 times (except for samples after 10 h and 12 h) and the residual citrate (as the carbon source) was determined by the test kit No. 139076 from Boehringer Mannheim GmbH, Mannheim, Germany.

Glucose

Glucose was used in one experiment replacing sodium citrate as the carbon source, maintaining the pH constant at the optimal level. The glucose analyzer from the Yellow Springs Instruments Inc., Yellow Springs, Ohio, USA was used to determine the residual glucose.

HPLC analysis

100 µl of the medium after separation of the cells was mixed with 400 µl HPLC-grade methanol and the extracellular amino acids determined by the OPA method. 1.0 ml lysed cell suspension was centrifuged. 100 µl supernatant was mixed with 400 µl HPLC-grade methanol and the intracellular amino acids analysed (lysis was by Triton X-100 as mentioned before). The detection limit of our HPLC system is 0.001 mM for most of the amino acids.

In all the points on the graphs where error bars are shown and the values on the table where deviations are indicated, the determinations were done in duplicate and the average value shown.

Results

Optimisation of the carbon and nitrogen sources

Of the 12 carbon sources tested at 18, 24 and 36 h of fermentation, sodium citrate showed the maximum activity at 24 h. The optimal concentration was found to be 1.0%. Nine simple and 12 complex nitrogen sources were tested at 18 h and 24 h of fermentation. Diammonium hydrogen phosphate at 0.16% was found to be the best nitrogen source. For reasons of space limitation, we will not show the results of these preliminary experiments.

Effect of glucose

Glucose, normally the preferred carbon source for L-asparaginase production, proved to be inferior during the optimization of the carbon nutrients. A study was made to determine the effect of addition of glucose at different levels and time on enzyme production, cell growth and pH of the medium. The medium contained basal mineral

salts plus diammonium hydrogen phosphate (0.16%) and glucose or sodium citrate singly or in combination. Table 1 shows the results. The replacement of sodium citrate by glucose as a single carbon source results in the lowering of the pH of the culture medium and lower production of the enzyme although cell growth is not affected (Table 1). However, the use of a combination of sodium citrate and glucose at lower concentration (0.5% each) raises the pH above 7.5 and gives an enzyme yield higher than that obtained from glucose only.

While comparing the effect of glucose and citrate on L-asparaginase and cellular protein production in the reactor, the pH of the medium was controlled at the optimum pH of 8.0. Figures 1 and 2 indicate that, although there is no significant difference in the rate of cellular protein production by replacement of citrate by glucose as a carbon source, the rate and amount of L-asparaginase production are considerably lower in the glucose medium. Figure 3 shows that glucose and citrate utilization are fairly rapid during the initial stage of the process, although the rate of utilization of glucose is much slower.

Effect of aeration and agitation

A maximum enzyme level of 1.2 IU/ml is obtained at 6 h with an aeration rate of 1.0 vvm and agitation of 700 rpm (Fig. 4) The peak value of cellular protein formation is, however, attained at 10 h under same conditions of aeration and agitation (Fig. 5). The data points and the error bars have been removed to improve the clarity. The maximum deviation from the mean values of Fig. 4 and Fig. 5 is $\pm 0.5\%$. The amount of cellular and enzyme protein synthesis is dependent upon the pO_2 level in the medium (Fig. 6). It is evident from Figs. 4, 5 and 6 that, under optimum conditions of aeration and agitation, the amount of total cellular protein and rates of cellular and enzyme protein formation are highest when the pO_2 level is near 60% saturation. Increasing the stirrer speed has a greater effect in increasing the dissolved oxygen concentration than the aeration rate. Thus, the presence of more dissolved oxygen has a significant effect in increasing L-asparaginase synthesis by *E. aerogenes*.

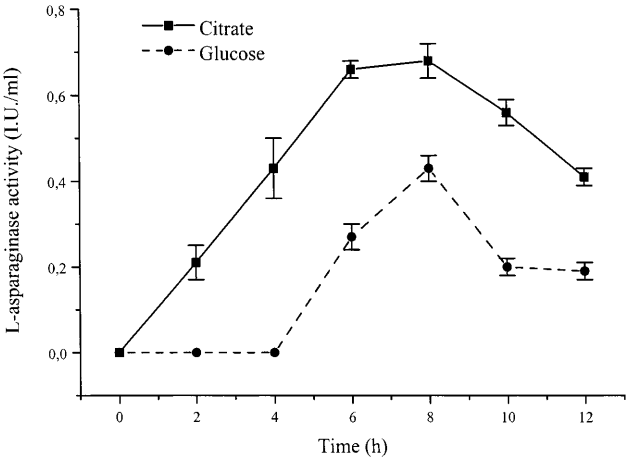

Fig. 2 Effect of glucose and citrate as carbon sources on L-asparaginase production by *Enterobacter aerogenes* in a 2.0-l bioreactor

HPLC analysis of the amino acids

Although 18 amino acids were detected intracellularly and extracellularly, only intracellular asparagine and extracellular alanine proved to be interesting. Asparagine was absent inside the cells which showed high L-asparaginase activity under our conditions. In the process where the L-asparaginase activity was low, e.g. cultivation with glucose as the carbon source, asparagine was found intracellularly. Extracellular alanine

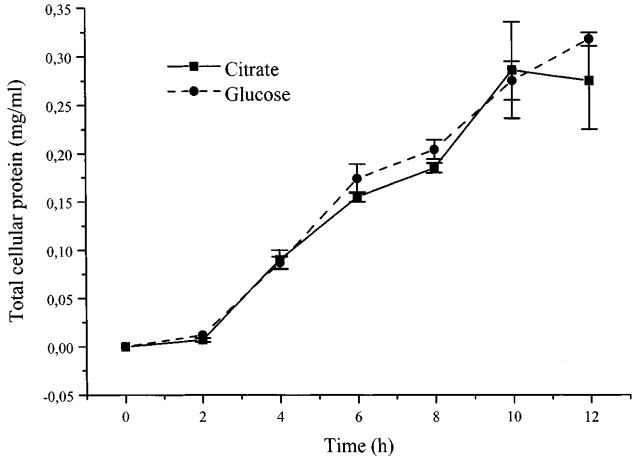

Fig. 1 Effect of glucose and citrate as carbon sources on growth (as cellular protein formation) of *Enterobacter aerogenes* in a 2.0-l bioreactor

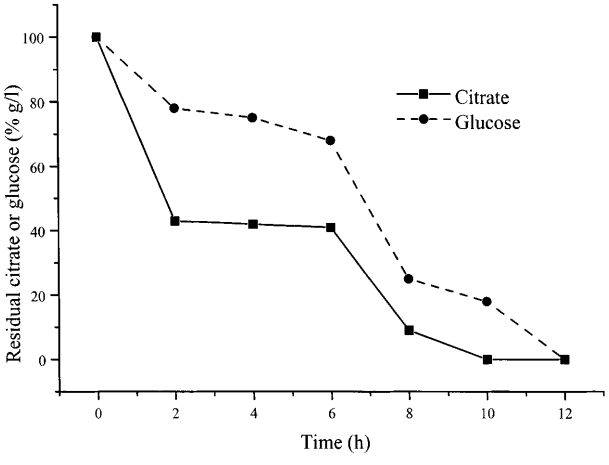

Fig. 3 Utilisation of glucose and citrate as carbon sources during L-asparaginase production by *Enterobacter aerogenes* in a 2.0- bioreactor

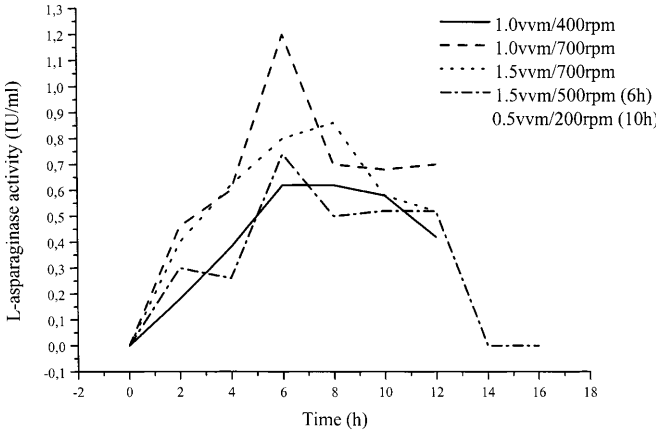

Fig. 4 Effect of aeration and agitation on L-asparaginase production by *Enterobacter aerogenes* in a 2.0-l bioreactor

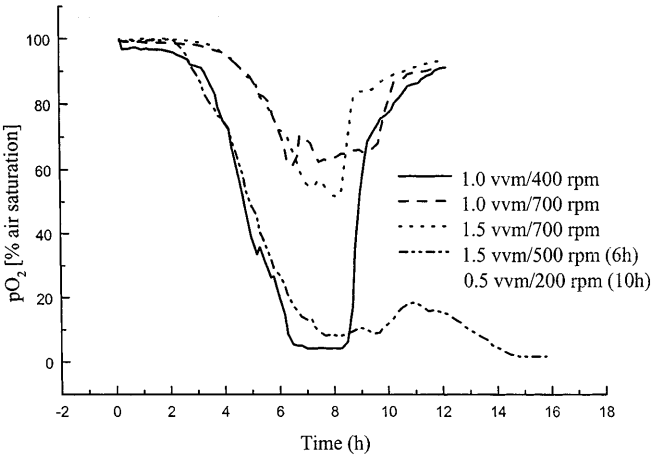

Fig. 6 Dissolved oxygen profiles during different aeration/agitation conditions during growth of *Enterobacter aerogenes*

showed an increase with time when the pO_2 value remained low for 10 h (Fig. 7).

Discussion

Sodium citrate (1.0%) and diammonium hydrogen phosphate (0.16%) have proved to be the most suitable carbon and nitrogen sources, respectively. It is interesting to note that some investigators (Tosa et al. 1971; Tyulpanova and Ermenko 1974) have shown the inferiority of sodium citrate as a carbon source for L-asparaginase production by organisms such as *Proteus vulgaris* and *Escherichia coli* 316/66.

The requirement of the ammonium ion for maximum enzyme synthesis in the medium containing $(NH_4)_2HPO_4$ is, however, interesting because this ion is a product of asparaginase action. This suggests the possible absence of nitrogen catabolite repression of the ammonium ion on L-asparaginase synthesis in *E. aerogenes*. The stimulatory effect of ammonium ions on L-asparaginase synthesis has been demonstrated in *Vibrio succinogenes* by Albanese and Kafkewitz (1978). The inhibitory effect the ammonium ion on enzyme production has been shown in bacteria such as *P. vulgaris* by Tosa et al. (1971), *Bacillus licheniformis* by Golden and Bernlohr (1985), *Bacillus subtilis* by Sun and Setlow (1991), and in yeast, *Saccharomyces cerevisiae* by Roon et al. (1982).

Glucose has proved to be a poor carbon source for the production of L-asparaginase by *E. aerogenes*. There is also a drop in the pH in glucose-containing medium. It is possible that the adverse effect of the acidic pH of the medium is responsible for the lower enzyme production when glucose is used as the carbon source. Glucose may also lower the enzyme yield by acting as a repressor. Repression of L-asparaginase synthesis by glucose has been shown in bacteria such as *Serratia marscences* (Heinemann and Howard 1969) *E. coli* (Barnes et al. 1977) and *P. vulgaris* (Tosa et al. 1971). Contrary observations have also been made on the enhancement of L-asparaginase synthesis by glucose in *Serratia marcescens* by Khan et al. (1970).

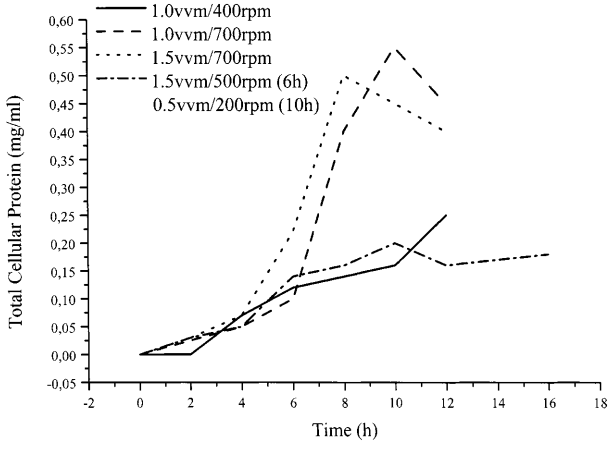

Fig. 5 Growth of *Enterobacter aerogenes* under different conditions of aeration and agitation in a 2.0-l bioreactor

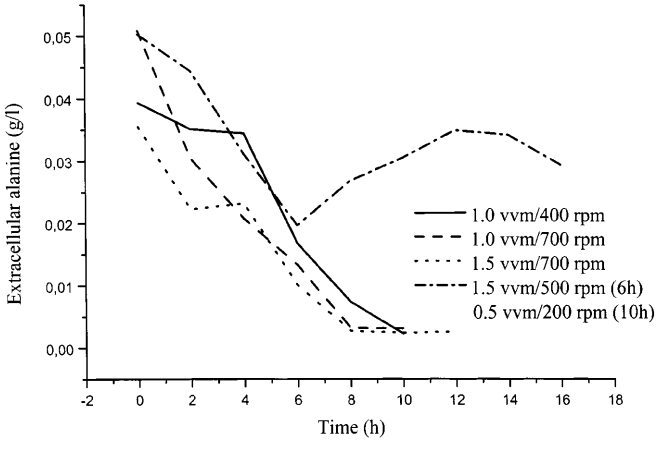

Fig. 7 Extracellular alanine formation under different conditions of aeration and agitation during growth of *Enterobacter aerogenes*

A study was, therefore, made to find out the effect of glucose where the medium pH was controlled at 8.0, which was found to be the optimum for L-asparaginase production. It was observed that there is lower production of the enzyme in the presence of glucose, although synthesis of cellular protein is not affected. This suggests that glucose acts as a repressor for L-asparaginase in *E. aerogenes*.

Investigations on the effect of aeration and agitation on L-asparaginase production show that maximum enzyme yield is obtained when the bacteria is supplied with sufficient amounts of oxygen. As the physiology of *E. aerogenes* is strongly influenced by the amount of dissolved oxygen in the medium (Byun et al. 1994; Mukherjee et al. 1999b), we consider the difference in the dissolved oxygen levels in the cultivations with air flow rates of 1.0 vvm and 1.5 vvm (stirrer speed 700 rpm) to be responsible for the significant differences in the enzyme activities under both conditions. In contrast, other workers have reported maximum production of L-asparaginase from *Erwinia aroideae* (Liu and Zajic 1973) and *Citrobacter* spp. (Bascomb et al. 1975) when the pO_2 dropped to zero during the process. It is evident from the results that the pO_2 is the limiting factor for L-asparaginase synthesis by *E. aerogenes*.

It was interesting to note that intracellular asparagine was absent in all processes which could be attributed to the high L-asparaginase activity when citrate was used as the carbon source. This observation has not yet been reported by other workers.

Detection of extracellular alanine under conditions of low pO_2 can be correlated to the shift of the metabolism of *E. aerogenes* from the aerobic to the fermentative metabolism. The pathway for aerobic breakdown of citrate in *E. aerogenes* was established by O Brien and Stern (1969). The presence of sodium ions in the medium led to the repression of α-ketoglutarate dehydrogenase and activation of oxaloacetate decarboxylase (which requires sodium ions for its activity), suggesting that the metabolism of citrate now proceeded via the fermentative pathway in cultures with low dissolved oxygen tensions. Alanine is derived by a single-step reaction from pyruvate, catalyzed by alanine amino transferase. Formation of pyruvate is characteristic of anaerobic metabolism, formed from the decarboxylation of oxaloacetate. It appears that under conditions of low pO_2 with excess pyruvate, alanine is produced in greater amounts than needed by the organism, and is thus excreted.

To conclude, this study shows that the biochemistry of L-asparaginase formation in *E. aerogenes* has similarities and dissimilarities not only to the other members of the broad group of Coliforms but also to other bacteria, yeast and fungi. In this regard, it would be of interest to test the biological activity from this new source of L-asparaginase.

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