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SHORT COMMUNICATION

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Amino acid supplementation enhances urokinase production by *HT-1080* cells

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Abstract Medium optimization is an important strategy that can lead to several fold increase in the production of proteins in cell culture. However, the usual methods of medium optimization are complex and time consuming. Urokinase is a widely employed thrombolytic drug for the treatment of stroke. We describe here medium optimization for maximizing urokinase production by HT-1080 cells using supplementation of specific amino acids. The new specifically designed method resulted in 240 % increase in urokinase productivity.

Keywords Urokinase · Production medium · HT-1080 · Amino acid supplementation

Introduction

An array of ready-to-use media mixes is available for the growth of a broad variety of cell types. However, when the primary objective of large-scale culture is to obtain a large amount of a specific protein, further optimization of culture medium has to be carried out. This is because nutrient depletion has been considered a major cause of the reduction of metabolic activity and protein production [1]. Examples of successful media manipulation leading to several fold increase in production of target biomolecule have been documented in recent literature [10].

Urokinase is one of the most important thrombolytic drugs used for the treatment of stroke and embolism. It has been established that high-density perfusion culture facilitates higher urokinase production in culture [4, 5]. The goal of the present work was to develop medium that (1) supports highly specific urokinase production; (2) maintains cell viability for prolonged culture duration; (3) simplifies downstream processing. Many investigators have reported that alleviation of amino acid limitation results in significant enhancement of productivity [6–8]. This is understandable since in the post-confluent period, the metabolic machinery is directed toward the production of the desired protein (urokinase) wherein amino acids are used as the building blocks. Supplementation of amino acid mixtures and protein hydrolysates has been shown to improve urokinase production by *HT-1080* cells substantially [3]. However, it was found that an excess of amino acids such as phenylalanine in the medium appeared to have a negative impact on urokinase production [3]. Moreover, higher concentrations of amino acid mixtures also had an adverse effect on production. Taken together, these results suggested that amino acids are limiting in the medium (DMEM/F12 1:1) and supplementation of specific amino acids can enhance the production. Therefore, in order to understand the amino acid requirements of *HT-1080* cells in culture, the consumption pattern of different amino acids in medium was determined. The data obtained were used to design a specific production medium that eliminated nutrient limitation and resulted in increased urokinase production from HT-1080 cells.

Materials and methods

Cell line and culture conditions

Human fibrosarcoma cell line *HT-1080* was procured from National Center for Cell Science, Pune (India). Dulbecco's

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minimum essential medium (DMEM/Ham F12 1:1) with 10 % (v/v) newborn calf serum (NBCS) from GIBCO (USA) was used for routine culture maintenance. The cultures were regularly monitored for absence of mycoplasma contamination using the Hoechst stain method. Viable cell concentration was determined by the trypan blue dye exclusion method. HT-1080 cells were cultured in 25 cm^2 tissue culture flasks at 37 °C in a 5 % CO_2 incubator.

Assay of urokinase activity

Urokinase amidolytic activity was assayed with urokinase-specific chromogenic substrate pyro-Glu-Gly-Arg-*para*-nitroanilide from Sigma USA using the method given by Tait et al. [9]. One unit of urokinase activity was defined as the amount that produces l mole of *p*-nitroaniline (pNA) from the chromogenic substrate in 1 min under standard reaction conditions (pH 7.4 and 37 °C).

Ten microliters of chromogenic substrate solution (stock concentration 0.1 mg/ml in Tris buffer, pH 7.4) was mixed with 70 μl of the Tris-HCl buffer and 50 μl of filtered culture medium. The mixture was incubated at 37 °C for 30 min; 150 μl of 10 % acetic acid was added to stop the reaction. The absorbance of the released pNA was measured at 405 nm using a microplate reader (Tecan, USA). The difference between the 30 min and zero min absorbance values gives an estimate of the amount of pNA released.

Amino acid concentration in the cell culture broth was determined by the method given by Chang et al. [2]. The method is based on pre-column derivatization of amino acids with 4-(dimethylamino)-azobenzene-4′-sulfonyl chloride (Dabsyl-Cl). The derivatized amino acids were determined by HPLC using the DABS customized cartridge for amino acid analysis from Grom® Analytik, Rottenburg-Hailfingen, Germany (200 mm × 4.6 m ID). Initially, culture samples were filtered onto a 0.45-μm membrane (Millipore, Bedford, MA, USA), and the filtrate was collected for amino acid analysis. The protein in the filtrate was removed by precipitation with trichloroacetic acid (TCA) (0.5 M final concentration) and then centrifuged at 5,000*g* for 15 min. The supernatant was filtered on Millipore membranes (0.45 μm) and subjected to a derivatization reaction for HPLC analysis.

Derivatization procedure

In a capped Eppendorf tube, the amino acid sample (5 μl) was diluted with 50 μl of 100 mM (pH 9) sodium carbonate buffer (Merck, Mumbai India). Subsequently, 100 μl of 1.3 mg/ml Dabsyl-Cl reagent (Grom® Analytik Rottenburg-Hailfingen, Germany,) in acetonitrile (Merck, Mumbai India) was added, and the reaction mixture was incubated at 70 °C for 20 min. The reaction mixture was cooled, and then 345 μl of sample dilution buffer consisting of 25 mM of phosphoric acid (Merck, Mumbai, India) and acetonitrile (Merck, Mumbai, India) in a ratio of 90:10 v/v was added to the reaction mixture. The pH of the sample dilution buffer was adjusted to 7.0 using solid sodium hydroxide.

HPLC

The column was operated at 1.0 ml/min and 45 °C using a UV-visible detector at 436 nm. The mobile phase consisted of Eluant A: 82 % v/v sodium acetate buffer (24 mM, pH 6.6), 18 % v/v acetonitrile and Eluant B: 40 % v/v acetonitrile, 60 % v/v 2-propanol 1.0 ml/min. All the buffer components and HPLC grade solvents were sourced from Merck, India. The linear gradient scheme used was: (Time: %B): (0–4 min), 5–20 % B (4–8 min), 20–25 %, B (8–15), 25–60 % B (15–27), 60–100 %,B (27–28 min), 100 % B (28–32 min).

Results and discussion

The cells were first allowed to grow to confluence (48 h) in the growth medium (DMEM/Hams F12 with 10 %v/v NBCS). After confluence, the growth medium was replaced with production medium. In the production medium, the serum concentration was reduced to 1 % v/v using DMEM/Hams F12 as basal medium. The spent medium analysis was performed to determine the amino acid limitations prevailing during urokinase production phase. The concentrations of amino acids and urokinase were determined in the

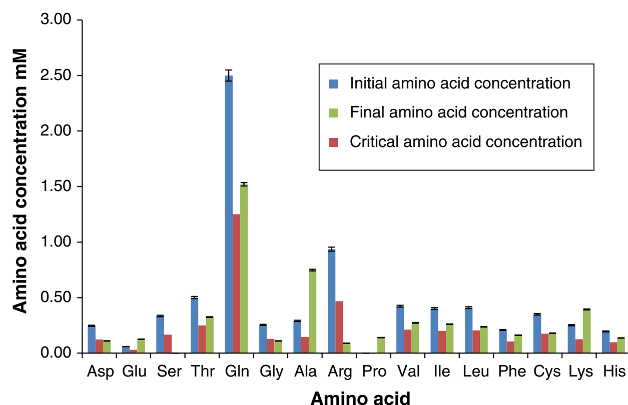

Fig. 1 Comparison of initial, critical and final concentration of amino acids in the cell culture broth at the end of 24 h of batch culture in unsupplemented medium

culture broth every after 12 h (Fig. 1). It was conjectured that the depletion of the amino acid concentration below the 'critical level' limits urokinase production. Critical amino acid concentration was defined as 'the concentration of amino acid in the medium below which urokinase production is limited.' Amino acids that were required to be supplemented to the basal were identified using the following inequality (Eq. 1)

$$C_{f,i}^{\text{Basal}} \subseteq \; xC_{0,i}^{\text{Basal}} \tag{1}$$

where $C_{0,i}^{\text{Basal}}$ and $C_{f,i}^{\text{Basal}}$represent the initial and residual concentrations of the ith amino acid in the medium at the end of 24 h of culture and $\left[xC_{0,i}^{\text{basal}}\right]$ is the critical amino acid concentration. For the present case, α was assigned a value of 0.5. Thus, only those amino acids that decreased below critical concentration (i.e., residual amino acid concentration ≤50 %) during the 24 h of batch culture satisfied the inequality (Eq. 1). These were selected for supplementation of the medium. Other amino acids were presumed to be present at non-limiting levels. A comparison of initial, critical and final concentrations of amino acids in the cell culture broth at the end of 24 h of batch culture has been illustrated in Fig. 1.

It was observed that the concentrations of serine, arginine, glycine and aspartic acid fell below the critical level in first 24 h. Approximately about 91, 80, 54 and 51 % of arginine, serine, glycine and aspartic acid, respectively, were consumed within initial 24 h of culture. Thereafter, owing to the biosynthetic activity of the cells, the concentration of the amino acids glycine and arginine were found to increase. The concentration of the amino acid serine continues to decreases till it is undetectable in the medium by 48 h. The concentration of amino acid aspartic acid does not decrease further after 24 h. Concomitantly, it was observed that the maximum urokinase production rate was obtained in the first 12 h of the production phase (Fig. 2). The rate of production declines considerably after the first 24 h. The amino acid consumption and urokinase production pattern obtained reveals the strong dependence of availability of amino acids and urokinase production.

Supplementation of the basal medium was carried out using the following relationship:

$$C_{0,i}^{\text{new}} = C_{0,i}^{\text{Basal}} + \left(yC_{0,i}^{\text{Basal}} - C_{f,i}^{\text{Basal}}\right) \tag{2}$$

Since supplementation of the medium would be expected to improve cell viability, y was chosen 10 % higher than x. If y was equal to 0.5, it would mean that exactly the consumed amount of a given amino acid would be supplemented; there is a risk that the concentration of this supplemented amino acid would fall below the critical concentration $[xC_{0,i}^{\text{Basal}}]$ in the subsequent culture, which would require that the procedure be repeated several times until the medium formulation with no amino acid limitation, had been achieved. With $y > x$, a nutrient formulation with no amino acid limitation was reached only in two steps.

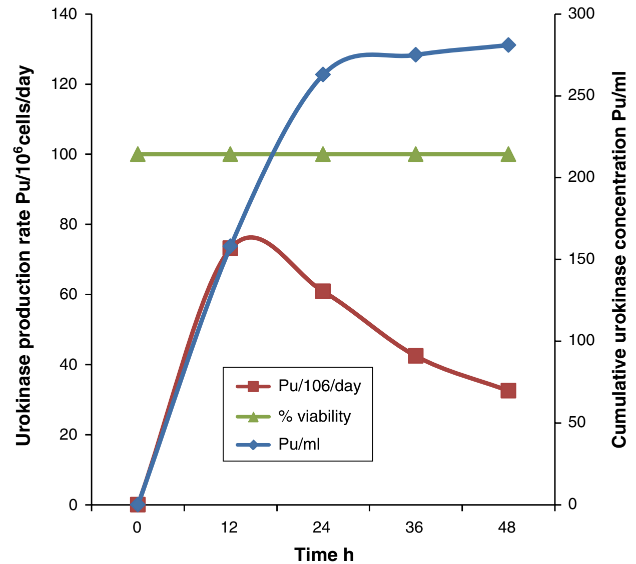

Fig. 2 Change in urokinase concentration and productivity in the production phase

Table 1 Growth parameters and urokinase production in enriched media

Parameter	Basal medium	Supplemented medium
$X_{\text{v max}}$ (cell/ml)	9×10^5	9.25×10^5
Urokinase$_{\text{final}}$ (PU/ml)	119.7	416.25
q_{uPA} (PU/10^{6}cell/day)	665	2250

The supplemented medium was formulated using the above correlations (Eqs. 1, 2) to compute the required quantities of limiting amino acids, i.e., arginine (78 mg/l), aspartic acid (1.2 mg/l), serine (30 mg/l) and glycine (200 mg/l) to the basal medium (DMEM/F12 1:1). Batch culture was then undertaken using the new medium formulation, and the amino acid concentrations were determined by HPLC. In the optimized medium, it was observed that amino acid concentrations were maintained above the critical level. This aided achieving a higher viable cell number and urokinase production in the supplemented medium (Table 1). The specific urokinase production rate increased four-fold in the production medium. Since the production of urokinase as well as cell viability is higher in the supplemented medium, the requirement of amino acid building blocks is also higher. This is reflected by larger produced/consumed amounts of amino acids in the supplemented medium.

Serine, arginine, glycine and aspartic acid are non-essential amino acids; nevertheless, it can be said that the depletion of these amino acids below the critical level puts the burden on the biosynthetic machinery of the cell. Glutamine is essentially required for the generation of energy for the mammalian cells and as an amino acid building block. In human kidney cells, the pathway for glutamine biosynthesis from arginine is active. Therefore, it could be expected that most of the arginine is utilized for generation of glutamine. The rest of the amino acids collectively amount to <50 % of the total amino acid uptake in the production phase. Therefore, with a simple two-step method the urokinase production medium was optimized. The optimized production resulted in an approximately 240 % increase in specific urokinase productivity.

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