Production of methionine by a multi-analogue resistant mutant of *Corynebacterium lilium*

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Abstract

Multi-analogue resistant mutants of *Corynebacterium lilium* were developed using UV and N-methyl-N'-nitro-nitrosoguanidine (NTG) mutagenesis in order to overproduce methionine. Although the methionine yield at each stage of mutation by UV and NTG mutagenesis were comparable, it was observed that the NTG mutants had higher cell growth and glucose utilization rates. From the parent NTG mutant *C. lilium* E 4 resistant to ethionine, the highest methionine producing strain *C. lilium* M-128 resistant to 3.5 mg ml\(^{-1}\) of ethionine, 3.0 mg ml\(^{-1}\) of norleucine, 3.8 mg ml\(^{-1}\) of methionine sulfoxide and 3.8 mg ml\(^{-1}\) of methionine methyl sulphoniumchloride was derived. When *C. lilium* M-128 was grown under optimised conditions, it produced 2.3 g l\(^{-1}\) of methionine in a 15 l batch reactor. The maximum cell mass concentration obtained was 17 g l\(^{-1}\) dry cell weight resulting in a cell yield coefficient of 0.34 g g\(^{-1}\). The dissolved oxygen concentration was controlled at 40% saturation by cascade control of the airflow rate and agitation speed.

Keywords: Methionine; *Corynebacterium lilium*; Mutagenesis; Submerged cultivation; Dissolved oxygen

1. Introduction

Methionine is an essential amino acid, required in the diet of man and other animals for normal growth and functions of the body. It is frequently deficient in plant proteins and this can sometimes lead to malnutrition in people dependent solely on plant products-based diet that is not well balanced. Such deficiencies can only be overcome by an exogenous supply of the essential amino acids. The discovery of microbial production of glutamic acid [1] eventually inspired the production of other amino acids from microorganisms. Microorganisms naturally produce the biologically active L-form of amino acids [2]. However, in the case of methionine, it is usually difficult to obtain a wild type strain capable of producing significant amount of this amino acid because it is biosynthesised via highly branched pathways under strict feedback regulation [3,4]. Hence, a mutant with genetically altered regulation mechanism needs to be developed for the production of methionine [5,6].

Analogue, particularly of amino acids, effectively function as true feedback inhibitors without participating in other useful functions in the cells [7,8]. Mutants resistant to analogues have altered and deregulated enzymes and such mutants in the absence of analogues can synthesise the corresponding metabolite in excess, which is eventually excreted into the medium [5,9].

It has been observed that strains of *Corynebacterium* or *Brevibacterium* have much simpler regulatory mechanisms (Fig. 1) than that of *Escherichia coli* [10]. This is presumably because these organisms evolved in an environment that was poor in amino acids. The major function of the regulation of amino acid biosynthesis in such an organism is to adjust its rate in response to the growth rate of the organism. Thus there is no need to adjust the ratios of the various amino acids produced. When such an organism produces excess methionine (to enhance growth), it is also likely to produce excess threonine, lysine and isoleucine. The major regulation of the branched pathway can then be achieved by a system in which only a few of the products inhibit the first
common enzyme as shown in Fig. 1. A number of organisms having a less complex regulatory mechanism, have been used for the production of methionine [11]. However, there is no report on \textit{C. litiun}.

One of the objectives of this work was to develop a multi-analogue resistant mutant of \textit{C. litiun} for the over production of methionine. In the present study, we present the details of the development of a regulatory mutant of \textit{C. litiun} for the production of methionine. The kinetics of methionine production by the mutant \textit{C. litiun} M-128, which is resistant to four methionine analogues, was studied in a 15 l reactor.

2. Material and methods

2.1. Microorganisms and maintenance

A wild type strain of \textit{C. litiun} was the parent strain used in this study. Mutants were developed from the wild type strain by both UV as well as \textit{N}-methyl-\textit{N}'-nitro-\textit{N}-nitrosoguanidine (NTG) induced mutagenesis. The details of UV mutagenesis are presented elsewhere [12,13] and the details of NTG mutagenesis are presented in the subsequent sections. The mutants were maintained on minimal media containing 10 g l\(^{-1}\) glucose, 6 g l\(^{-1}\) urea, 0.3 g l\(^{-1}\) MgSO\(_4\), 1.0 g l\(^{-1}\) K\(_2\)HPO\(_4\), 0.1g l\(^{-1}\) KH\(_2\)PO\(_4\), 20 g l\(^{-1}\) biotin, 4.8 mg ml\(^{-1}\) ethionine, 3.3 mg ml\(^{-1}\) norleucine, 3.8 mg ml\(^{-1}\) methionine sulfoxide, and 3.8 mg ml\(^{-1}\) L-methionine methysulphoxide, and stored at 4 °C.

2.2. Mutagenesis and development of single analogue resistant mutants

To obtain methionine analogue resistant mutants from wild type strain of \textit{C. litiun}, the minimum inhibitory concentration for various analogues was determined by plating cells on minimal agar plates containing different concentrations of analogues. Both NTG and UV induced mutagenesis was performed to develop analogue resistant mutants. The wild type strain was grown in Luria broth medium for 24 h, centrifuged, washed with citrate buffer (pH 5.0) and subjected to mutagenesis until 99.9% killing was achieved. Cells of \textit{C. litiun} were introduced into 500 µg ml\(^{-1}\) of NTG solution in citrate buffer and incubated in rotary shaker at 37 °C for 5 h. The cells were centrifuged, washed twice with phosphate buffer (pH 7.5), diluted suitably and transferred to the minimal plates and incubated at 30 °C for 2 days. Single isolated colonies of the mutants were transferred to the plates containing methionine analogues in increasing order of concentration. Cells grown on the plate containing highest concentrations of analogue were selected and tested for methionine production initially in shake flask culture.

2.3. Development of multi-analogue resistant mutants

Several thousand mutants were obtained during the course of development of multi-analogue resistant mutants. This large number was reduced to about 500 potential mutants by transferring cells to analogue containing plates using replica plating technique. Single isolated colonies of the mutants were then transferred separately and simultaneously to plates containing ethionine, norleucine, methionine sulphoxide and methyl methionine sulphonium chloride. A grid was constructed manually on the base of each Petri plate, in which each square of the grid was numbered and inoculated with an isolated colony. A colony selected by replica plating was inoculated on to the squares bearing the same number in each plate. Only those colonies that could grow in all the plates of one set were transferred to the next set of plates containing a higher concentration of the analysed analogue.
concentration of each analogue. The process was repeated several times until the number of mutants was reduced to between 60 and 70.

2.4. Media and culture conditions

A loopful of cells from a slant was transferred into a flask containing minimal medium (without analogues) and allowed to grow for 24 h at 30 °C and 150 rpm in a rotary shaker. The reactor was inoculated with 5% (v/v) of this. The medium used for screening mutants in shake flask cultures and for the production of methionine in a 15 l reactor (B-Braun Biostat-C) were the same and contained 5% glucose, 0.6% urea, 0.3% MgSO₄, 0.1% K₂HPO₄, 0.01% KH₂PO₄, and 20 μg l⁻¹ biotin. The pH of the medium was adjusted to 7.0 using H₂SO₄ before inoculation. The flasks were incubated at 30 °C and 150 rpm for 48 h. Batch reactor studies on methionine production were carried out using the mutant strain C. illium M-128. In the reactor, the pH was controlled at 7.0 and the temperature at 30 °C by PID controllers. The dissolved oxygen (DO) concentration was maintained at 40% saturation by cascade control of agitation and aeration. The pH, temperature, DO, airflow rate and agitation speed were monitored online by a data acquisition system. The methionine, cell mass and glucose concentrations were measured off-line.

2.5. Analytical methods

Growth was determined based on optical density (OD) measurements at 600 nm. The dry cell weight (in g l⁻¹) was obtained as 0.3 x OD from a standard curve constructed for this purpose. Methionine in the fermented broth was estimated by the spectrophotometric method [14] and glucose was estimated by using glucose analyser (Accutrend Sensor, Roche Diagnostics, USA).

3. Results and discussion

3.1. Methionine production by various analogue resistant mutants of C. illium

A number of mutants of C. illium, resistant to methionine analogues, have been obtained by UV and NTG mutagenesis. The highest methionine producer obtained by UV mutagenesis, C. illium NTE 99, resistant to norleucine, triazole and ethionine, yielded 1.85 mg ml⁻¹ methionine. The genealogy of the mutants is shown in Fig. 2. Results indicate that UV [12,13] and NTG mutants at each stage of development (Fig. 2) produce comparable amounts of methionine. Since chemical and physical mutagenesis methods are not specific, mutant strains isolated tend to contain many other unrelated mutations, some of which may have adverse effects on the organism. It has also been observed in the present study that some mutants, in spite of being resistant to methionine analogues, had lost their methionine producing ability after further mutation.

The method outlined in Section 2.3 has been followed to select mutants having a good potential of over-producing methionine. This method saves a substantial amount of time since the resistance of the mutant to various levels of each analogue can be determined simultaneously in each step. The selected mutants were subcultured several times in minimal media followed by culturing in the presence of analogues to test their stability. It was observed that UV mutants tend to be less stable than NTG mutants.

For NTG mutagenesis, a number of mutants initially resistant to 1.2 mg ml⁻¹ of ethionine were derived from wild type strain. Among these, one of the stable and high yielding mutants C. illium E-4, was found to produce 500 μg ml⁻¹ of methionine in shake flask cultures. This compares well with the report of Yamada et al. [6] who obtained 420 μg ml⁻¹ methionine by an ethionine resistant mutant OE 120 derived from obligate methylobacterial bacteria, Methylophonas sp. OM 33. C. illium E-4 was subjected to further mutagenesis to obtain a better methionine producer. Mutants obtained in the second phase of mutagenesis were selected by growing on ethionine and norleucine containing minimal agar plates, separately in increasing order of concentration. A number of mutants, grown to the highest ethionine and norleucine containing plates were selected and tested for methionine production in shake flask culture. The stability and productivity of these mutants were checked for several generations and C. illium NE 57 was selected as a mutant having the potential for subsequent mutagenesis. C. illium NE 57 was resistant to 2.0 mg ml⁻¹ of ethionine and 1.5 mg ml⁻¹ of norleucine, and produced 840 μg ml⁻¹ methionine. After primary screening of mutants obtained from C. illium NE-57 by mutagenesis, only 51 mutants resistant to 3.0 mg ml⁻¹ ethionine, 2.5 mg ml⁻¹ norleucine and 3.5 mg ml⁻¹ methionine sulphone were selected and tested for methionine production and stability. The results of a comparative study of some selected mutants, producing more than 1 mg ml⁻¹ methionine in shake flasks are given in Table 1. Out of these mutants, C. illium M-29 produced 1.86 g l⁻¹ methionine in shake flask culture in 48 h. This compares well with the 1.850 g l⁻¹ methionine produced by the UV mutant C. illium NTE 99 (Fig. 2). Chattopadhyay et al. [15] have also reported that a mutant of Escherichia coli K-12 developed by them, resistant to methionine analogues (norleucine, ethionine and α-methylmethionine) and threonine analogue (α-amino-β-hydroxy valeric acid) produced 2 mg ml⁻¹ of both methionine and threonine. However, the mutants developed by them,
resistant to only the methionine analogues ethionine and 5-bromouracil, produced 1 mg ml⁻¹ of both methionine and threonine.

In the next stage, *C. lilium* M-29 was subjected to further mutagenesis. After the initial screening, 63 mutants resistant to 3.8 mg ml⁻¹ ethionine, 3.5 mg ml⁻¹ norleucine, 3.8 mg ml⁻¹ methionine sulfoxide and 3.8 mg ml⁻¹ methionine methylsulphonium chloride were selected and tested for methionine production in shake flask. One stable mutant *C. lilium* M-128 was obtained that produced 1.98 mg ml⁻¹ methionine in shake flask culture. Kase and Nakayama [2] also developed a threonine auxotrophic quadra-analogue resistant mutant of *Corynebacterium glutamicum* ESLMR-724, having resistance to selenomethionine, trifluoromethionine, 1,2,4-triazole and methionine hydroxamate that produced 2 mg ml⁻¹ methionine. Since *C. lilium* M-128 is not a threonine auxotroph, a higher methionine yield may be expected if a threonine or lysine auxotroph is derived from it.

Optimising the medium composition and process parameters can increase methionine yield further. Banik and Majumdar [16,17] reported the first detailed study of the effect of medium constituents, minerals and composition on the productivity of methionine by mutant strains. They derived a mutant of *Micrococcus glutamicus* X1 by ethyl methane sulphonate and X-ray for mutagenesis, producing 3.0 mg ml⁻¹ methionine; the yield of methionine was further improved to 4.5 mg ml⁻¹ by optimising the composition of the media.

### Table 1
Methionine production, growth and glucose consumption by mutants M 25, 28, 29, 35, 41, 48

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Fermentation parameters</th>
<th><em>C. lilium</em> mutants</th>
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<tr>
<td></td>
<td></td>
<td>M 28</td>
</tr>
<tr>
<td>24</td>
<td>Methionine (mg l⁻¹)</td>
<td>170.0</td>
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<td></td>
<td>Residual glucose (g l⁻¹)</td>
<td>32.18</td>
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<td></td>
<td>Cell mass (g l⁻¹)</td>
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<td>36</td>
<td>Methionine (mg l⁻¹)</td>
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<td></td>
<td>Residual glucose (g l⁻¹)</td>
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<tr>
<td></td>
<td>Cell mass (g l⁻¹)</td>
<td>5.86</td>
</tr>
<tr>
<td>42</td>
<td>Methionine (mg ml⁻¹)</td>
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<td></td>
<td>Residual glucose (g l⁻¹)</td>
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<tr>
<td></td>
<td>Cell mass (g l⁻¹)</td>
<td>6.16</td>
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<td>48</td>
<td>Methionine (mg ml⁻¹)</td>
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<td>Residual glucose (g l⁻¹)</td>
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</tr>
<tr>
<td></td>
<td>Cell mass (g l⁻¹)</td>
<td>6.17</td>
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Sharma [13] performed a medium optimisation based on a statistical experimental design and achieved a 30% improvement in a batch reactor over shake flask cultures. The optimised medium contained 2% glucose, 0.6% urea, 0.3% MgSO₄, 0.1% K₂HPO₄, 0.01% KH₂SO₄. Further, Sharma and Gomes [12] determined for the first time the optimum DO concentration for the production of methionine. Through a series of CSSTR experiments they demonstrated that the optimum DO for both cell mass and methionine productivity was 40% of the saturation level at a dilution rate of 0.17 h⁻¹. The optimised medium proposed by Sharma [13] was initially used as the running medium. Later it was observed that the NTG mutants had a higher capacity to utilize glucose and a concentration of 5% glucose was experimentally ascertained to give the best results.

3.2. Kinetic studies in a 15 l batch reactor

Submerged cultivation of C. illium M-128 was carried out in a 15 l reactor (B-Braun Biostat-C) equipped with automatic control and data acquisition system. Five experiments of methionine production were carried out to study the kinetics of the process. The reactor was controlled at a temperature of 30 °C and pH 7 by the in-built controllers. The DO was controlled at 40% saturation by cascade control of airflow rate and agitation speed. These conditions were previously optimised for studying the kinetics of UV mutants [12]. The cell mass, residual glucose and methionine concentrations were measured off-line. The temperature, pH, DO, airflow rate and agitation speed were monitored on-line. The average cell mass, residual glucose and methionine concentrations for the five experiments are shown in Fig. 3 and the performance of the cascade oxygen controller for one of the batch reactor experiments is presented in Fig. 4.

The batch experiments were carried out for 58 h and the maximum concentration of 2.3 mg ml⁻¹ of methionine was obtained after 48 h of fermentation. The maximum specific growth rate of C. illium was 0.18 h⁻¹ and the cell yield coefficient Yₓₘ was 0.34 g g⁻¹. The value of the maximum specific growth rate was close to the value of optimum dilution rate of 0.17 h⁻¹ determined at 40% saturation DO in the CSSTR experiments with UV mutants [12]. Therefore, controlling the DO does appear to control the physiological state of the cells, which in turn affects both the cell growth and methionine productivity. Further, these results indicate that a DO concentration of 40% saturation is optimum for methionine production and is not restricted by the kind of mutation the microorganism has undergone.

One of the characteristics of the NTG mutants was that the cell mass concentration at the end of 48 h of submerged cultivation was about 17 g l⁻¹ which was twice that obtained for UV mutants. The glucose utilization capability was not adversely affected in these mutants since they were able to utilize almost 50 g l⁻¹ in 48 h.

From the online profiles of DO, airflow rate and agitation speed (Fig. 4), it is clear that the energetic demand of C. illium M-128 begins within 2 h of the start of the batch culture. The airflow rate begins to increase until it reaches a maximum of 18 lpm. At this point the agitation speed starts to increase and changes to control the DO at the set point of 40%. After about 24 h the cell growth reached its stationary phase (Fig. 3) and the decrease in the demand for oxygen was reflected by the gradual drop in the agitation and airflow rate values (Fig. 4).

Fig. 3. Methionine production, growth and glucose utilisation by C. illium M-128 showing the concentration profiles for five batch experiments. Run 1 (+), Run 2 (○), Run 3 (×), Run 4 (△), Run 5 (□). Average trend for five batches (—).
3.3. Some observations on altered regulation of methionine biosynthesis in C. lilium M-128

The extent of inhibition and repression of the mutant C. lilium M-128 was also checked. The mutant C. lilium M-128 and wild type C. lilium were grown separately in the minimal media containing methionine, lysine or threonine at two different concentrations, 2 and 5 mg ml\(^{-1}\). Although the mutant C. lilium M-128 could grow well in both media and a cell mass concentration of about 6.0 g l\(^{-1}\) was obtained after 36 h, the wild type strain did not grow in either medium. Failure of the wild type to grow in the presence of 2.0 mg ml\(^{-1}\) threonine and lysine was presumably due to the shut down of aspartokinase that made the microorganism incapable of producing methionine required for its growth, whereas no growth in the presence of 2 mg ml\(^{-1}\) methionine was presumably due to the inhibition of homoserine dehydrogenase, making the microorganism incapable of producing threonine and isoleucine (Fig. 1). In Corynebacterium and Brevibacterium sp., threonine inhibits homoserine dehydrogenase individually and aspartokinase in the presence of lysine (Fig. 1). Therefore for the overproduction of methionine it is essential for a mutant to have an alteration in regulatory site of aspartokinase along with homoserine dehydrogenase. The mutant C. lilium M-128 probably had an alteration in aspartokinase such that the altered allosteric regulatory site of the enzyme had lower affinity for lysine and threonine; the growth of the mutant in the presence of 5 mg ml\(^{-1}\) of lysine and threonine seems to support this hypothesis. A paper chromatography test showed that this mutant did produce minute amounts of lysine and threonine. Efforts are underway in this laboratory to develop a threonine auxotrophic mutant from C. lilium M-128 to further enhance the production of methionine.

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References


