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Improved Microbial Production of Lysine by Developing a New Auxotrophic Mutant of *Corneybacterium glutamicum*

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Abstract: The objective of this investigation was to develop a new auxotrophic mutant from a regulatory mutant and to examine its potency for lysine production. Glutamate-homoserine-alanine triple auxotrophic mutant MRLH-GHA$_{10}$ was derived from thiosine resistant mutant of *Corneybacterium glutamicum.* Its potency was examined in various media containing different carbon sources. The most potent auxotrophic mutant developed in this investigation produced 38, 33 and 28.5 g L^{-1} lysine, in the media containing glucose, molasses and starch hydrolyzate, respectively as carbon sources in a stirred tank fermenter, at 30 °C and at 500 rpm agitation.

Key words: *Corneybacterium glutamicum*, regulatory mutant, auxotrophic mutant, potency, lysine production

Introduction

A major segment of population of the technologically underdeveloped countries depends on cereal grains, which lack some of the essential amino acids including lysine. Amino acid supplementation involving only lysine, tryptophan and threonine will, without exception, increase the quality of proteins of edible cereals to make them comparable to a standard milk protein casein (How *et al.*, 1965). Lysine alone increased markedly the quality of protein of sesame flour (Joseph *et al.*, 1962). To prevent the development of protein deficiency disease, amino acid supplementation is a prerequisite. For this purpose rapid production of lysine is needed worldwide. Previously, lysine was obtained from protein hydrolyzate; then chemical and enzymatic methods were followed; at present great attention has been given to microbial production. Among the microorganisms, bacteria, especially the *Corneybacterium glutamicum,* have sufficient productivity. However, the wild strains of this bacterium could not accumulate lysine due to the concerted feedback inhibition (Fig. 1). A lot of work has been done on auxotrophic and regulatory mutant of glutamate producing bacteria for the production of lysine. This investigation focuses on the development of auxotrophic mutant from a regulatory mutant, which in addition to homoserine and alanine, is also a glutamate negative mutant. Its potency for lysine production was examined in different carbon sources, such as glucose, molasses and starch hydrolyzate.

Materials and Methods

Media composition: The experimental work was performed at the Department of Biological Sciences, Quid-e-Azam University, Islamabad and at the Department of Chemistry, Gomal University, Dera Ismail Khan during 1998-2001.

The Complete Medium used in this investigation contained 1.0g beef extract, 1.0g bactopeptone, 2.0g glucose, 0.3g sodium chloride and 100ml of distilled water. Two grams of agar was used as solidification agent. The pH of the medium was 7.2. The Minimal Medium of Davis and Mingioli (1950) was used to grow cells for Ultra Violet (UV) irradiation.

Fermentation Medium-1 (FM 1) was composed of 1.0g glucose as a carbon source, 2.5g ammonium sulfate, 0.1g potassium dihydrogen phosphate, 0.05g magnesium sulfate heptahydrate, 2.0g calcium carbonate, 0.2g ferrous sulfate heptahydrate, 0.2 mg manganese chloride tetrahydrate, 80 mg glutamate, 0.02 mg homoserine, 0.02 mg alanine, 0.005 mg d-biotin, 0.02 mg thiamine HCl and 100 ml distilled water. The pH of the medium was 7.5.

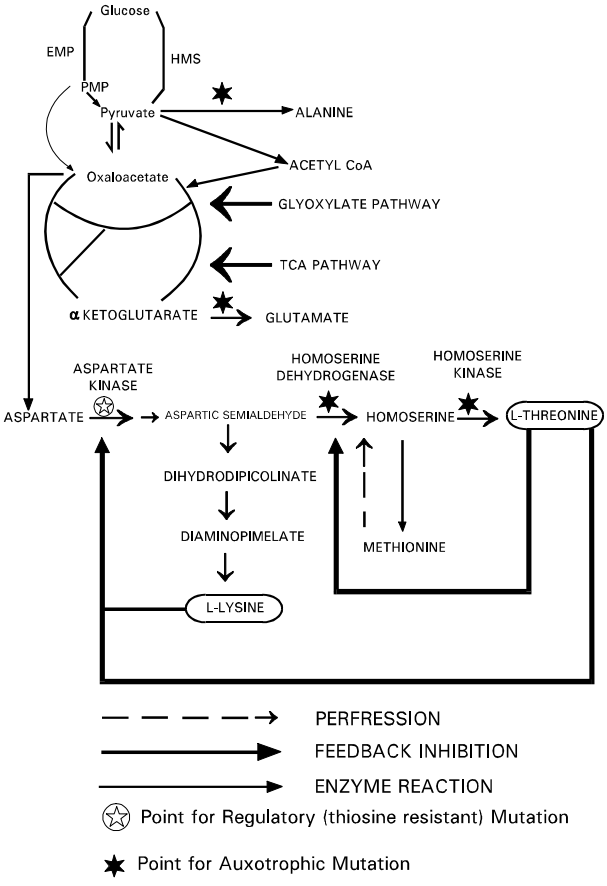

Fig. 1: Biosynthetic Pathway of Lysine in *Corynebacterium glutamicum*

Fermentation Medium-2 (FM 2) had similar composition as that of *FM 1*, except that molasses was used as a carbon source instead of glucose.

Fermentation Medium-3 (FM 3) had similar composition as that of FM 1, except that starch hydrolyzate was used as a carbon source instead of glucose.

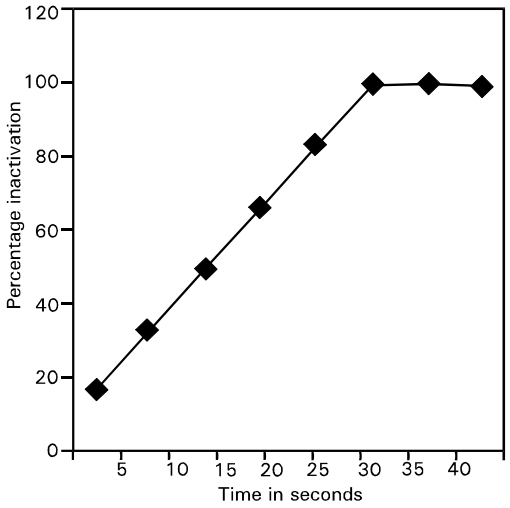

Fig. 2: Effect of UV Dosage on percentage inactivation

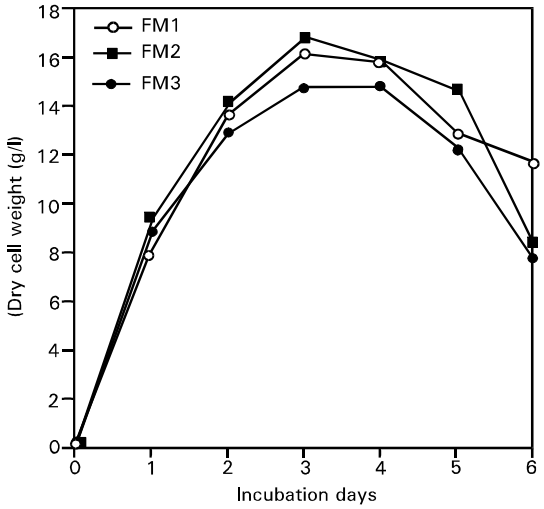

Fig. 4: Time course of dry cells weight production in different media

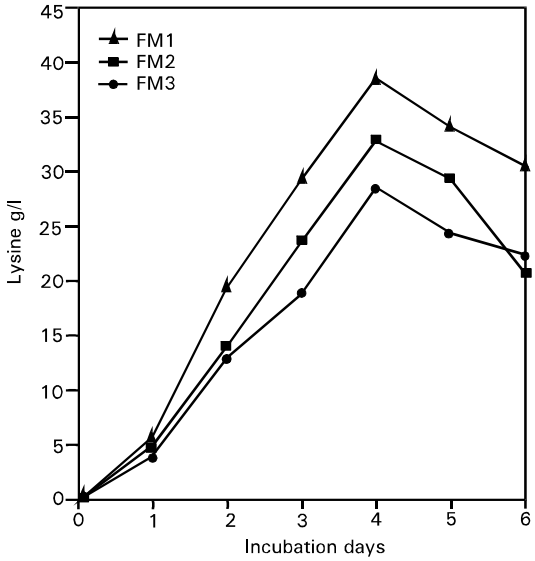

Fig. 3: Lysine production as influenced by fermentation media and incubation

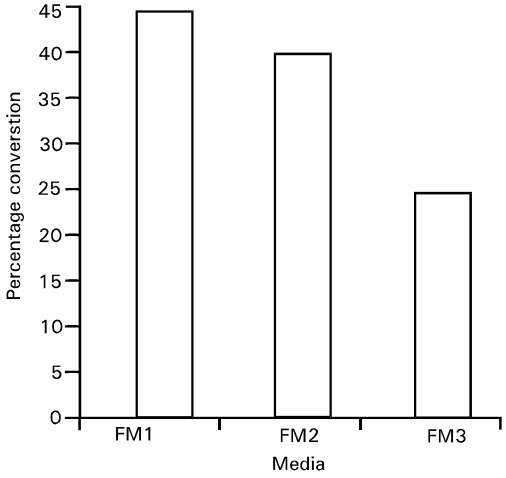

Fig. 5: Percentage conversion in different media

Culture methods

Screening of mutants for lysine production: Each of the fermentation media (20ml) in a 100ml flask was inoculated with loop of thialysine resistant mutants (MRLH-GHA$_{10}$) from 24hrs old complete agar plate and incubated on a reciprocal shaking incubator at 28 °C for 72hrs at 100rpm. Samples of all the mutants were centrifuged and the supernatant was collected for qualitative and quantitative examination of the lysine.

Batch fermentation in a stirred tank: Three liters of sterilized fermentation medium was taken in a 6 liter stirred tank fermenter. The fermentation was performed after inoculation with 300ml seed medium under 500 rpm agitation and 1.5 vvm aeration, at 30 °C for 6 days. One ml of broth was taken out with sterilized pipettes at definite intervals. The pH of the broth was controlled at 7.5, using 20 % ammonium hydroxide. Tween-20 was used as antifoaming agent.

Mutational treatment

Development and isolation of auxotrophic mutants: Exponentially growing cells (2x10^8 cells/ml) of thialysine resistant mutants were irradiated by UV radiations for 30 seconds under the same set of parameters as mentioned for the development of thialysine resistant mutants (Shah and Hameed, 1999). One ml of the UV irradiated cells was taken separately in each of two 10ml test tubes which were incubated in a reciprocal shaking incubator at 28 °C. After 14 hrs 50 units of penicillin G was added into each test tube. After another 5 minutes interval, 100 units of penicillinase was incorporated into each test tube followed by the addition of 1ml solution containing 0.04 % of each glutamate, homoserine and alanine. The contents in the test tubes were mixed thoroughly and then incubated under the above mentioned parameters. After 7hrs, 0.2ml of the culture from each test tube was spread separately on the complete agar media and then incubated again until the colonies of thialysine resistant mutants

were developed. Samples from each of the colonies were then streaked on minimal agar and also on the glutamate-homoserine-alanine containing minimal agar. Colonies that did not grow on minimal agar and grew only on glutamate-homoserine-alanine containing minimal agar were isolated. Each sample of the auxotrophic mutants was then separately cultured in 20ml auxotrophic screening media in 100ml flasks and incubated at 28 °C at 100rpm agitation. After 72 hrs, the samples were taken and centrifuged. The supernatant was collected for the qualitative and quantitative analysis of lysine.

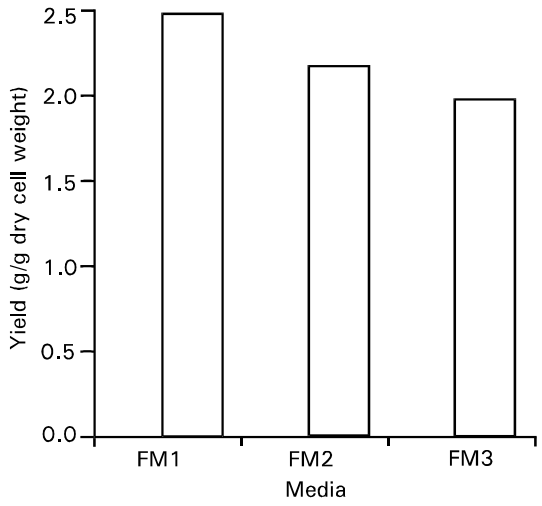

Fig. 6: Yield g/g dry cell weight using different media

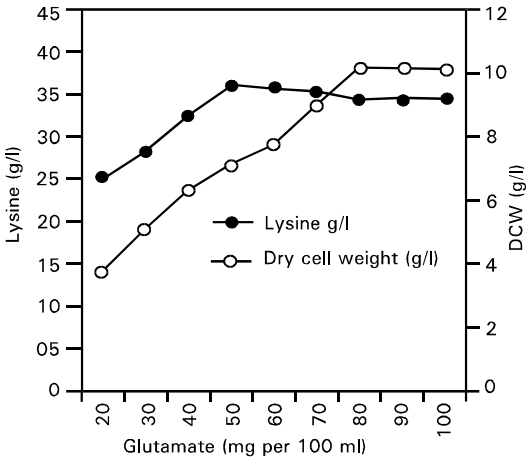

Fig. 7: Effect of glutamate on lysine production

Qualitative and quantitative analysis: For the qualitative analysis of lysine, paper chromatography (Momose and Takagi, 1978) was used; while the quantitative estimation of lysine was performed by acidic ninhydrine copper reagent method (Aiko *et al.*, 1971; Hsiao and Glatz, 1996).

Clarification of molasses: The molasses (1.5g) was diluted with distilled water (1 part ml), the pH of the mixture was adjusted to 9.5 and the mixture was placed in a shaking bath at 50 °C for 10 min. Then it was kept undisturbed overnight, at room temperature so that the solid material was sedimented. Later on the supernatant was removed and filtered.

Dry cells weight (DCW) achievement: After centrifugation, a few ml of 1M HCl was poured into the precipitate of bacterial cells and calcium carbonate in order to dissolve the later. The remaining bacterial cells were washed with distilled water and dried at 100 °C until a constant weight of the dry cells was obtained.

Results

Effect of UV dosage on lethality (Percentage inactivation): Generally the required percentage inactivation (lethality) level is 90-99.9 % (Sikyta, 1983). Exposure time must be such as to give the desired percentage inactivation. The exposure time was determined by studying the effect of various doses of UV light on $2x10^8$ cells/ml and $2x10^5$ cells/ml, respectively. Percentage survival after 30sec UV treatment was 99.9 % and 30 seconds were found as the optimum exposure time for UV treatment (Fig. 2).

Isolation of glutamate-homoserine-alanine triple auxotrophic mutants: Thiosine resistant mutants were treated with UV radiation for 30sec, followed by penicillin enrichment procedure. A total of 500 colonies were tested for glutamate-homoserine-alanine triple auxotrophs, among which 200 (40 %) colonies were screened for lysine production. The most potent mutants developed in this investigation produced 38, 33 and 28.5 g L^{-1} lysine, in the media containing glucose, molasses and starch hydrolyzate, respectively. Hence they were selected for further studies of lysine production in different media.

Lysine production: The most potent mutant MRLH-GHA_{10} was examined in media containing different carbon sources such as glucose, molasses and starch hydrolyzate. About 38.4, 33.0 and 28.6g lysine was accumulated in one liter of FM1, FM2 and FM3, respectively. The production profiles of lysine, DCW, conversion of carbon source to lysine and the yield/g DSW was also determined. Lysine production increased up to the 4^{th} day of fermentation and then gradually decreased in all the media. The maximum yield found was in FM1 (Fig. 3). DCW was increased up to the 3^{rd} day of fermentation, then remained constant for some time and afterwards it decreased significantly (Fig. 4). The conversion of the consumed carbon source to lysine was 43.8, 39.0 and 24.7 % in FM1, FM2 and FM3, respectively (Fig. 5). The yield/g DCW was 2.4, 2.0 and 1.9g in FM1, FM2 and FM3, respectively (Fig. 6).

Effect of glutamate on lysine production: Lysine production increased in a glutamate concentration dependent manner. The maximum yield was observed at glutamate concentration of 80mg/100ml. The cells mass was increased up to 50mg/100ml of glutamate and then became constant (Fig. 7).

Discussion

The biosynthetic pathway for lysine is known (Nakayama, *et al.*, 1985; Yamaguchi *et al.*, 1986). In *Corneybacterium glutamicum*, threonine and lysine stop the activity of aspartate kinase by concerted feed back inhibition, due to which the synthesis of lysine and threonine stops. For the microbial production of lysine, changes in the biosynthetic pathway are necessary for the blockage of undesirable side products.

Mutagenesis is a phenomenon by which changes can be introduced in the metabolic process. The most direct and general method for over production is the genetic removal of the feed

back control. The blockage of homoserine synthesis results in the release of the concerted feedback inhibition. Consequently, aspartic semialdehyde proceeds to lysine through the lysine synthetic pathway.

Different mutagens can be used for the desirable changes in the genetics of the strains of interest (Jacobson, 1981). UV light has been recommended as mutagen of the first choice. The ratio of mutation is usually very high and UV light is a relatively safe mutagen for the experimenters (Bridge, 1976). Thiosine resistant mutant did not produce enough lysine due to the concerted feed back inhibition, as mentioned above. Therefore, the blockage of other side reactions including conversion of pyruvate to alanine, α-ketoglutarate to glutamate and homoserine to threonine, are also necessary. These blockages were obtained by generating glutamate-homoserine-alanine triple auxotrophic mutant (Shah and Hameed, 1999).

The frequency of occurrence of auxotrophs by simple UV radiation is usually very low. So enrichment with penicillin-G after UV treatment was carried out. Penicillin-G sterilizes the actively growing cells by blocking the cell wall synthesis; thus only auxotrophic mutants survive after this treatment. Destroying penicillin with penicillinase allowed the growth of auxotrophs in the supplemented media containing glutamate, homoserine and alanine. As a result glutamate-homoserine-alanine triple auxotrophs were achieved. This technique has been used to isolate auxotrophs of both prokaryote and eukaryote (Saito and Ikeda, 1957).

Increase in lysine production without any change in cells mass above 50 mg/100 ml glutamate showed that 50mg is the nutritive requirement of the auxotrophs and above 50mg is only used for amination of oxaloacetate by glutamate aspartate transaminase. Additionally, glutamate also aminates the intermediate N-succinyl-α-amino-ketopimelate (Nakayama, *et al.*, 1985). Yamaguchi *et al.* (1986) reported that aspartate, which is the precursor of diaminopimelate is formed through a transamination reaction between glutamate as amino donor and oxaloacetate as amino acceptor. Kinnoshirta *et al.* (1960) reported that a mutant of *Corneybacterium glutamicum*, which are glutamate negative and possess glutamic acid dehydrogenase but lacks isocitrate dehydrogenase, catalyzes the production of α-ketoglutarate in TCA cycle. Ishino *et al.* (1984) reported that glutamate dehydrogenase had no meso α,ϵ-diaminopimelate dehydrogenase activity. The work of Ishino *et al.* (1984) and Yamaguchi *et al.* (1986) led to the conclusion that both diaminopimelate and diaminopimelate-dehydrogenase bipathways exist in the *Corneybacterium glutamicum*.

After screening, glutamate-homoserine-alanine triple auxotroph was examined for lysine production. When glucose was used as a carbon source maximum yield was achieved as compared with molasses and starch hydrolyzate. Nakayama *et al.* (1985) also used molasses as a carbon source for *Corneybacterium glutamicum*. Such hydrolyzate is a cheaper carbon source and lysine production from this has been studied only to a limited extent. Moreover, *Corneybacterium glutamicum* could not hydrolyze starch, therefore starch hydrolyzate was used. In the molasses medium lower concentration of lysine was observed than that of the glucose medium, due to the inhibitory effect of molasses on growth and production. Plachy *et al.* (1988) observed decreased lysine production and cells mass after substitution of sugar by molasses in mutant of *Brevibacterium flevum*. Starch hydrolyzate have relatively low content of mono-saccharides, therefore the production of lysine was the least. Pelechova *et al.* (1983) obtained 10-12g/L lysine by using paper hydrolyzate as carbon source.

A new auxotrophic mutant (Glutamate-homoserine-alanine triple auxotrophic mutant MRLH-GHA$_{10}$) was derived from a thiosine resistant regulatory mutant, *Corneybacterium glutamicum*. The mutant produced in this investigation showed higher potency of lysine production in the media containing various carbon sources such as glucose, molasses and starch hydrolyzate. However, glucose was found to be the best carbon source regarding the production of lysine by the said mutant.

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