Development of Threonine Auxotrophic Mutant from Thialysine Resistant Mutant for Improved Lysine Production

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Summary: *Corynebacterium glutamicum* was isolated in our microbiology research lab. Thialysine resistant mutants were developed by treating with ultraviolet radiation. The most potent resistant mutant was further treated by U.V radiation. As a result 42 different threonine-auxotrophic mutants were developed, isolated and screened for lysine production. The most potent mutant produced 18.3 mg/ml lysine in shake flask fermentation after 6th day of incubation (temp.30°C, 200 rpm, pH 7.5). The lysine production increased to 26.2 mg/ml in stirrer tank fermenter on 4ht day of fermentation (temp 30°C, pH 7.4, agitation 500 rpm, aeration 1 vvm).

Introduction

Lysine is an essential amino acid not biologically synthesized in the body. Cereal and plant protein tends to be rather deficient in lysine. It is reported that lysine is the first limiting amino acid in virtually every cereal grain known to man. [1]

Nakayama *et al.*, [2] clarified the mechanism of L-lysine accumulation by *Micrococcus glutamicus* (Syn. *Corynebacterium glutamicum*) and proved that in the prototroph, the aspartokinase would be subjected to concerted feed back inhibition by intracellular levels of threonine and lysine. These inhibitions may prevent the formation of excessive amount of aspartyl phosphate and thus aspartic semialdehyde and the amino acids as threonine, lysine and methionine derived from it. The effect of various amino acids on aspartokinase reaction on the biosynthetic pathway of lysine have also been studied by Nakayama *et.al.* [2] and found that neither threonine nor lysine exerted an inhibitory effect when added singly to the system, but prominent inhibition was noted by combined use of threonine and lysine. Homoserine in combination with lysine was not inhibitory. The degree of inhibition of aspartokinase was about 50% at the concentration of 10 micro moles of each threonine and lysine per ml. It remained in the same level even at higher concentration of lysine and threonine.

In the present study, metabolic controls on lysine were bypassed by the development of thialysine resistant mutant of *Corynebacterium glutamicum*, and threonine auxotrophic mutant was developed from thialysine resistant mutant, isolated and examined for lysine. In the said auxotrophs, the pathway is blocked between threonine and aspartic semialdehyde. Therefore, threonine can not be synthesized. Lysine is accumulated when threonine in the medium is limited. Thus, it continues to synthesize aspartic semialdehyde leading to lysine.

Results and Discussion.

Effect of UV Dosage on Lethality Level (Percentage Inactivation.)

Generally, for mutation the required level for percentage inactivation (lethality level) is usually 90-99% [3]. Exposure time must be such as to give 99.9% inactivation. The exposure time was determined by studying the effect of various UV

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We feel tremendous pleasure and honor to dedicate our manuscript to Prof. Dr. Atta-ur-Rahman, the hero and the pride of the teachers and scientists of Pakistan, on the auspicious occasion of his 60th birthday. We pay him our befitting tributes for his meritorious services as a teacher, scientist and as a Minister of Science and Technology. His services will be remembered for long time in the years to come.

We pray for the very long life of this outstanding scientist.

Dr. Abdul Haleem Shah and Dr. Gul Majid Khan

dosages on $2x10^8$ cells/ml (Fig 1). Viable count before and after UV treatment was $2x10^8$ cells/ml and $2x10^5$ cell/ml, respectively.

Percentage survival, after UV treatment for 30 sec was 0.1 and the percentage inactivation after 30 sec. of treatment was 99.9%. So, 30 sec was the optimum time of exposure for UV treatment.

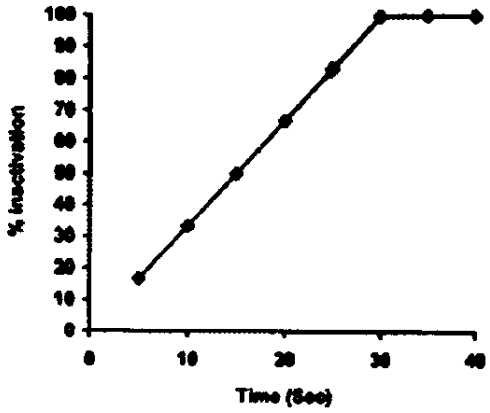

Fig. 1: Effect of Ultraviolet dosage on % inactivation of corynebacterium glutamicum (n=3)

Growth Inhibition by Thialysine.

Thiosine is an analogue of lysine [4], therefore, it acts as a false feedback inhibitor of lysine production. It is also clear from the biosynthetic pathway of lysine that in *Corynebacterium glutamicum*, lysine and threonine inhibit the aspartokinase activity [5]. The growth inhibiting effect might be promoted by the addition of threonine. So the effects of various concentrations of thiosine alone and in combination with threonine were studied on the growth of the said bacteria.

Minimal medium (5ml) was inoculated by exponentially growing cells at concentration of $2x10^5$ cells/ml in 15 ml test tube and incubated in reciprocal shaking incubator (28°C, 100 rpm). The turbidity (OD at 600 nm) was estimated at the time when the unsupplemented controlled tube showed maximum turbidity (Fig.2). The turbidity of the controlled tube was 13 at 14 hrs, by taking optical density at 600nm. (Fig. 2) shows the relationship between the growth and the concentration of supplemented thiosine and thiosine plus threonine. Complete inhibition was not observed by thiosine alone at concentration of 3mg/ml. Complete inhibition was observed by

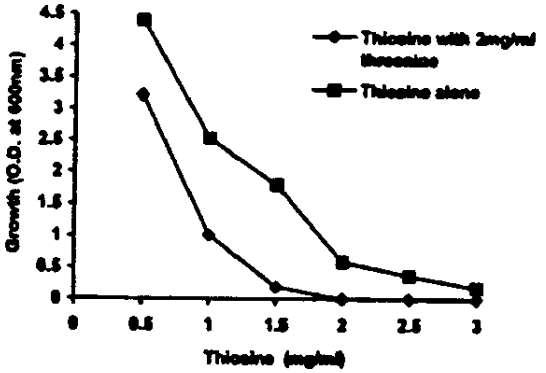

Fig. 2: Effects of different concentrations of thiosine and thiosine with threonine on the growth of corynebacterium glutamicum (n=3)

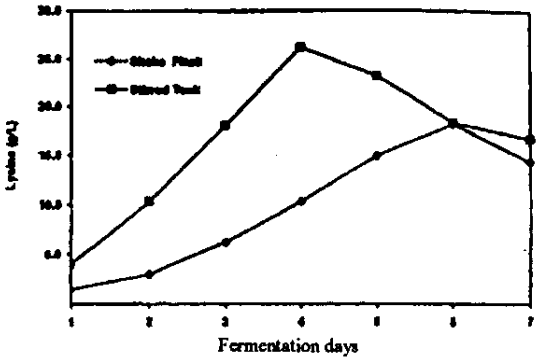

Fig.3: Lysine Production in Shake Flask and Stirred Tank Fermentation (n=3)

2mg/ml concentration of thiosine and threonine. Total eight resistant colonies were appeared per plate when 0.5 ml of UV irradiated cells were spread per plate on minimal agar plus thiosine and threonine, so the percentage of resistant colonies was 8×10^{-3}.

Lysine Production

Thialysine resistant mutant produced 8 g/L lysine in fermentation medium while the threonine auxotrophic mutant, derived from thialysine resistant mutant, produced 18.3 g/L lysine in shake flask and 26.2 g/L lysine in stirrer tank fermentation. The lysine production increase upto 6th day of fermentation and then decreased. In stirred tank, lysine production increased upto 4th day of fermentation and then decreased (Fig 3). In shake flask, the dry cell weight increased upto 4th day, then remained constant that afterward decreased. In stirrer tank, the dry cell weight increased upto 3rd day, then remained constant and finally decreased. (Fig. 4)

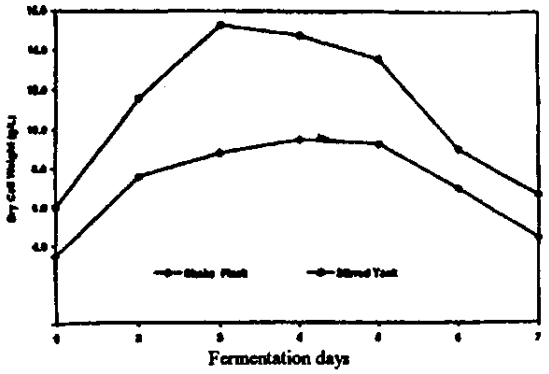

Fig. 4: Dry Cell weight produced in Shake Flask and Stirred Tank Fermentation during Lysine production (n=3)

Lysine is the primary metabolite of microorganisms. Thiosine is an analogue of lysine, which acts as false feedback inhibitor, as a result inhibits the synthesis of lysine due to which the growth also stops. Mutagenesis is a mean by which change can be introduced in the metabolic pathway. Different mutagens can be used for the desirable change in the genome of the strain of interest [6]. UV light has been recommended as a mutagen of the first choice. The ratio of mutation to lethality is high and is a safe mutagen for the experimenter. For mutation, 90-99% inactivation is required [7]. In this study in *Corynebacterium glutamicum* 99.9% inactivation (lethality level) was achieved after 30 sec. at exposure distance of 15cm from UV lamp. The percentage of resistant colonies were $8x10^{-3}$.

Penicillin inhibits a transpeptidation reaction required to crosslink the peptidoglycan component of the cell wall of most prokaryotes. Penicillin reacts covalently with a small number of different proteins (penicillin-binding proteins) located in the membranes of these microorganisms [8]. Some of these proteins exhibit transpeptidase activity. The treatment of cells with Penicillin after U.V radiation sterilizes only the actively growing cells (Prototroph) by blocking their cell wall synthesis. However, it does not affect the auxotrophic cells, as they only survive but do not grow. These auxotrphic cells can be rescued from a solution of penicillin by first destroying the penicillin and then establishing environmental conditions suitable for the growth. To achieve more auxotroph U.V irradiated cells were grown in a minimal medium containing penicillin so that protrophs initiate active growth and as a result were killed by penicillin. Auxotrophs present in the population can not initiate growth because of the absence of required nutrients (threorinine) and as a result these non-growing cells survive. Penicillin was then destroyed by penicillinase [8, 9]. Finally, threorinine required for theorinine auxotroph was added and the cell were incubated so that a dense population of cells, now containing a greater percentage of auxotrophs, was obtained.

As it is stated earlier threorinine auxotroph was derived from thialysine resistant strain which had both the properties of regulatory mutants (thialysine resistant) and threorinine auxotrophic mutants and these mutants produced 26 g/L lysine then thialysine resistant mutant which produced 8 g/L lysine. The reason is that in thialysine resistant mutants the aspartokinase are insensitive to feedback inhibition, that is it will produce more lysine as well as threonine. Because no more threonine is available which can cause concerted feed back inhibition. So the aspartic semialdehyde leads only to lysine synthesis. As a result, more lysine was accumulated than produced by thialysine resistant mutant. Nakayama *et al.*, [2] patented the strain of *Brevibacterium flavum* having a requirement of threonine and resistant to feed-back inhibition of lysine and threonine.

In any fermentation process, yield was higher in fermenter than in shake flask culture because in fermentation pH was maintained at 7.5 while in shake flask it was not constant. Even the triple auxotroph (methionine- threonine- alanine) of the same resistant produced 23.4 gram per liter lysine in shake flask [10]. Further more higher biomass was observed in stirrer tank than in shake flask.

Experimental

Complete medium used had the following composition per 100 ml distilled water. Beef extract 1g, Bactopeptone 1g, glucose 2g, sodium chloride 0.3g, pH 7.2. For solidification 2g. agar was used.

Minimal medium of Davis and Mingioli [11] was used to grow cells for UV radiation.

Fermentation medium had the following composition per 100ml distilled water. Carbon source (glucose) 10g, ammonium sulphate 2.5g, potassium dihydrogen phosphate 0.1g, magnesium sulphate seven hydrate 0.05g, calcium carbonate 2g, ferrous sulphate seven hydrate 0.2g, manganese chloride tetrahydrate 0.2 mg, d-biotin 5 microgram, thiamine HCl 20microgram. pH 7.5

Culture Method

Screening of Mutants for Lysine Production.

Fermentation medium (20ml) in 100ml flask was inoculated by loop of thialysine resistant mutants from 24hrs old complete agar plate and incubated on reciprocal shaking incubator at 28°C for 72 hours at 100 rpm. Sample of all mutants were centrifuged, the supernatant was examined for lysine production both qualitatively and quantitatively.[11]

Batch Fermentation in Stirred Tank.

Sterilized 3 liter fermentation medium was taken in 6 liter stirred tank fermenter. The fermentation was started after inoculation with 300ml seed medium under 500 rpm agitation and 1.5vvm aeration at 30°C for six days. One ml of the broth pipetted out with sterilized pipette at intervals. The pH of the broth was controlled automatically at 7.5 with 20% ammonium hydroxide. For antifoaming tween-20 was used.

Mutational Treatment

Development and Isolation of Thialysine Resistant Mutant

Stock cultures were maintained on agar slant of complete medium. Loop of activated cells were transferred to 20 ml minimal medium in 100 ml flask and incubated over night (20hrs) at 28°C on reciprocal shaking incubator at 100 rpm. Then 1 ml of the culture was diluted by 40 ml of the same fresh medium and incubated under the same parameter for 5 hrs, to obtain exponentially growing cells. The culture was diluted 10 times by minimal medium (minus glucose). Then 10 ml (2×10^8 cells/ml) of the cells suspension was taken in petriplate agitated on mini shaker at 10 rpm at exposure distance of 15cm from UV germicidal lamp. Ultraviolet radiation was applied for 30 sec. using a 30 watt UV germicidal lamp at exposure distance of 15 cm. The UV irradiated cells (0.5 ml) were spread on minimal agar plus 2 mg/ml each thiosine and threonine and incubated. Thiosine resistant colonies were picked up and screened for L-lysine by transferring a loop of each resistant colony to 20 ml screening medium in 100 ml flask on reciprocal shaking incubator at 28°C for 72 hrs. Samples were taken and centrifuged. The supernatant was examined for L-lysine.

Development and Isolation of Auxotrophic Mutants

For the development and isolation of auxotrophic mutant, the principle of Davis [12] Lederberg and Zinder [13] was followed. Exponentially growing cells (2x10 cells/ml) of thialysine resistant mutants were irradiated by U.V for 30 sec. under the same parameter as discussed for development of thialysine resistant mutants. One ml of U.V. irradiated cells were incubated in a 10 ml test tube (in duplicate) on reciprocal shaking incubator at 28 °C. After 14 hrs, 50 units of penicilline G was added and after 5 minutes 100 units of penicillinase was added then one ml of solution of threonine containing 0.04% threonine was mixed and incubated under the same conditions. After 7 hrs, 0.2 ml of the culture was smeared on complete agar and incubated. Each colony was then streaked on minimal agar and minimal agar containing threonine. Colonies that did not grow on minimal agar and grew only on minimal agar containing 0.02% threonine were isolated. Each auxotroph was then separately cultured in 20 ml auxotrophic screening medium in 100 ml flask and incubated at 28°C at 100 rpm. After 72 hrs, samples were taken and centrifuged. The supernatant was examined for lysine production.

Analysis of Lysine

For the qualitative analysis of lysine paper chromatography Momose and Takagi [14] was used. which are as follows:

Whatman No. 1 filter paper (19cm x 10cm) was used. Solution (10 µl) of standard and supernatant of the sample (Unknown) were spotted by mean of micro capillary tube at 3 cm from the bottom. The spots were air dried at room temperature and the chromatograms were developed by ascending method in butanol-acetic wate (40 : 10 : 50). Then 1.5 cm of paper was dipped in the solvent and was allowed to run up to 18 cm from the bottom. The paper was air dried, then uniformly sprayed with 0.5% ninhydrin in 95% acetone.

After drying in current air the paper was kept at 65°C for 30 minutes in the oven. The position of the amino acids were indicated by the formation of a well defined coloured zone. Thus the amino acid present in unknown sample were easily identified by reference to the zone of known amino acid.

Quantitatively lysine was estimated by acidic ninhydrin copper reagent method [15, 16] as follows:

Fermentation broth was clarified by centrifugation and 0.1 ml of the clear solution was mixed with 4.9 ml of distilled water. It was acidified

with 0.02 ml of 1 NHCl to pH 3.0 and added 0.1 g of charcoal with vigorous agitation to absorb color material in the diluted broth. The resultant solution was cleared without any color. To 0.2 ml of the clear solution, 0.5 ml of acidic ninhydrin-copper reagent was mixed and heated in boiling water for 20 minutes. After cooling in water, 2.5 ml of distilled water was added and the optical density was measured at 470 nm. Standard solution was assayed as above without charcoal absorption.

Dry Cell Weight (DCW)

After centrifugation, few ml of one MHCl was poured into the precipitate of bacterial cells and calcium carbonate to dissolve calcium carbonate. The remaining bacterial cells were washed with water and dried at 100°C until constant weight.

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