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L-lysine Production by the Homoserine Auxotrophic Mutant of *Corynebacterium glutamicum* in Stirrer Fermenter

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Abstract.- Homoserine auxotrophic mutants were developed from a local strain of *Corynebacterium glutamicum* (PCSIR-BI4) By using N-methyl-N-nitro-N nitrosoguanidine (NTG) as a mutagen. The developed mutants were screened for the production of lysine in cane molasses based fermentation medium. The lysine over producing mutant was designated as CGH-15. Different initial sugar concentrations of cane molasses were employed to obtain better lysine production in fermentation medium by the selected homoserine auxotroph. The optimal sugar level was found to be 60 gl^{-1} after 120 hours of incubation at 30°C, which enhanced lysine production from 4.00 to 25.05 g^{-1}. All the kinetic parameters including yield coefficients and volumetric rates revealed the overproduction of lysine by the mutant using black strap molasses as a sole source of carbon.

Key words: *Corynebacterium glutamicum*, lysine, fermentation, homoserine auxotrophs.

INTRODUCTION

Lysine is an essential amino acid for both human and animal. Majority of amino acids are commercially produced by fermentation process. *Corynebacterium* has been widely used for industrial production of lysine (Eggeling and Sahm, 1999; Herman, 2003; Ikeda and Nakagawa, 2003). The discovery and successful mutation of several microorganisms *(Corynebacterium glutamicum, Brevibacterium flavum, B. lactofermentum)* capable of producing significant quantities of this amino acid led to a rapid expansion of the amino acid industry during 1970s (Hirose and Shibai, 1980).

Lysine production occurs with classical mutants, which have been derived by many rounds of undirected mutagenesis and screening for increased productivity. Now a days, auxotrophic and regulatory mutants are being used for enhanced production of lysine. Auxotrophic mutants are defective in some way in a particular metabolic pathway and consequently lack or are deficient for a particular metabolic pathway's end product. Homoserine auxotrophs are deficient in homo serine dehydrogenase activity (Ozaki and Shiio, 1983) and can not produce homoserine for their growth. Therefore, homo serine is supplied in optimum quantities in the medium and all the pool of DL-aspartic-ß-semialdehyde (ASA) is directed for lysine production. ASA is a common precursor for homo serine and lysine biosynthesis (Miyajima *et al.*, 1968).

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Agro-Industrial by-products are being used as nitrogen and carbon source in lysine production (Ekwealor and Ebele, 2003). Sugarcane molasses is a cheap carbon source, containing sucrose, glucose and fructose at a total carbohydrate content of 50 to 60% (Reed, 1982). Glucose, fructose and sucrose are important carbon sources that have a pronounced effect on kinetics and stoichiometry of lysine production by *C. glutamicum* (Kiefer *et al.*, 2002). It provides a source offermentable sugars as well as some elemental nutrients, which playa key role in the fermentation process.

In the present work a homo serine auxotroph was developed from a locally isolated strain of *C. glutamicum* (PCSIR-BI4) by chemical mutation. Effect of different sugar concentrations was observed on lysine production by the mutant in a molasses this amino acid led to a rapid expansion of the amino acid industry during 1970s (Hirose and Shibai, 1980).

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In the present work a homo serine auxotroph was developed from a locally isolated strain of *C. glutamicum* (PCSIR-BI4) by chemical mutation. Effect of different sugar concentrations was observed on lysine production by the mutant in a molasses based medium. Kinetic parameters were estimated to study the fermentation behavior of the parent strain and the best lysine producing mutant.

MATERIALS AND METHODS

Microorgan

The locally isolated strain of *C. glutamicum* for the development of Homoserine auxotrophic mutants. All (PCSIR-BI4) was used mutants obtained after mutagenization, were screened for L-Iysine production. Maximum lysine producing strain that was CGH-15, selected for further studies.

Development and screening of mutant

Exponentially growing cells of the parent strain harvested in TYG broth of the composition g^{-1}: Tryptic soy digest, 10; yeast extract, 5; glucose, 5 and NaCl, 5. The pH was adjusted to 7.2±0.4. Vegetative cells were serially diluted to 10_{10} with 0.l M phosphate buffer. To 0.8 m1 of cell suspension, 0.2 m1 of 0.1 M phosphate buffer containing 2mg N-methyl-N-nitro-N-nitrosoguanidine (NTG) was added and incubated for 30 min. at 30°C. After mutagenic treatment cells were spread on TYG agar plates. Colonies appeared during 7 days of incubation were transferred on TYG agar plates with and without homo serine. Cultures appearing on the medium with homoserine, and exhibiting to growth on the medium without homo serine, were selected as homo serine auxotrophs. Developed mutants were than screened (all mutants were grown in the medium for 72 hours and broth after centrifugation, tested for lysine) for lysine production using TYG broth in shake flasks.

Clarification of molasses

Cane molasses was clarified (Panda *et al.,* 1984). After neutralization with calcium hydroxide solution (1.55%), the sugar concentration was maintained at required levels. The clarified cane molasses was kept under dark in a sterilized UV-chamber (Perkins Elmer, USA).

Fermentation conditions

A 2L Fermenter (Eyla, Japan) equipped automatic control of DO and pH was used. TYG medium was used as inoculum medium. For maintenance of the culture same medium was used with 20 gl^{-1} of agar. The pH was adjusted to 7.2-7.4. Fermentation process was carried out at 500 rpm, pH 7 and temperature at 30°C for 120 hours in fermentation medium of the following composition: Clarified cane molasses with reducing sugar adjusted to 20-100 gl^{-1}, 0.7 mg ml^{-l} homo serine, 40 g $(NH_4)_2SO_4$, 20g $CaCO_3$, 2g NaCl, 0.4g $MgSO_4$, 0.5g KH_2PO_4 and 1g K_2HPO_4. The pH was maintained at 7.0. The DO level was kept at 7 ppm by the pure oxygen enriched air and pH was kept at 7±0.2 with 50% NH_4OH throughout the cultivation.

Determination of kinetic parameters

Kinetic parameters were determined by the methods of Lawford and Rouseau (1993).

Analytical methods

Fermented broth was centrifuged at 10,000 rpm for 10 minutes. Supernatant was treated by derivatization with phenyl isothiocyanate and filtered with membrane filter of 0.2 !lm pore size before injecting and separated on a PICO. TAG column (150 by 3.9 mm, Waters, Milford, MA, USA) using an HPLC system (Waters). Lysine detection was carried out by UV absorbance at 230 nm (Hua *et al.,* 2000). Cell mass was measured by weighing the dry cell matter after drying at 105°C to constant mass. Sugar was estimated by DNS method (Miller, 1959). All the experiments and analysis were carried out in triplicates.

RESULTS AND DISCUSSION

Isolation and screening of homoserine auxotrophs

Parent culture of *C. glutamicum* (PCSIR-B14) was mutated with NTG. After mutation, cell suspension of the culture was spread on fermentation medium plates. Colonies obtained after 5 days of incubation were grown on the medium with and without homo serine. Cultures that grew only on homo serine containing medium and showed no growth in the medium without homo serine were selected as homo serine auxotrophs. All those mutant strains were then screened for lysine production in fermentation medium in 250ml Erlenmeyer flasks on a rotary shaker at 30°C and 150 rpm. Fifty seven colonies were obtained after mutation, out of which only 17 were found to be homo serine auxotrophs (Table I).

The developed mutants were screened for lysine production. The mutants produced different concentrations of lysine in a range of 9-21 gl^{-1} (Table I). The homo serine auxotroph designated as CGH-15 produced maximum lysine (21.792 gl^{-1}). Which was in good agreement with the work reported by Sano and Shiio (1967). Furthermore, CGH-15 increased lysine product from 4.0 gr10fthe parent strain to 21.792 gl^{-1}. Seto and Harada (1969) also observed increased production of lysine by homo serine auxotrophs of *C. acetophilum.*

It was evident from the results mentioned in Table I that product yield was maximum for CGH-15. The yield of cell mass was also found maximum for the selected mutant strain than other mutants and the parent strain. On the other hand, sugar consumption was minimum for the selected strain.

Effect of sugar concentration on lysine fermentation by CGH-15 mutant

The effect of different sugar concentrations (20-100 gl^{-1}) on lysine production by a homo serine auxotrophic mutant CGH-15 of the *C. glutamicum* was studied (Fig. 1). The maximum amount of lysine (25.052 gl^{-1}) was obtained in the medium containing 60 gl^{-1} sugar. The consumption of sugar and production of dry cell mass were 53.959 and 23.803 gl^{-1}, respectively. At 80 gl^{-1} sugar concentration, maximum cell mass production was observed although lysine production was lesser than at 60 gl^{-1} sugar. Therefore, this concentration promoted the cell mass production and not to the lysine production. Lysine and cell mass production were decreased at 20, 40 and 100 gl^{-1} sugar. Pham *et al.* (1993) also observed decreased production of lysine at higher and lower concentrations of glucose. Sugar concentrations significantly affect the lysine production as observed by Kiefer *et al.* (2002).

Time profile of lysine fermentation

Fermentation of lysine by the mutant strain CGH-15 was carried out for 120 hours. Time profile of lysine fermentation has been presented in Figure 2. About 87% of the cell mass was produced within first 24 hours. Further increase in cell mass was very slow and only 3 gl^{-1} increase was observed in further 96 hours. It was determined from the results that exponential phase of cell growth of *C. glutamicum* was completed within 24 hours of fermentation. Almost 55% of the sugar was consumed during that phase (growth phase). Consumption of sugar was slow in the following time period. Only minor quantities of lysine were produced in first 24 hours. The production rate was maximum between 48 to 72 hours, after which it almost stopped (production phase). Pham *et al.* (1993) and Matos and Coello (1999) also observed that maximum lysine production in 48 to 72 hours and lysine fermentation was completed in two different time phases; first, physiologic state of growth and second, lysine production phase. They also observed that most of

the carbon source was consumed in the first phase of fermentation.

Table I.- Lysine production by the parent and mutant strains in shake flasks.

Sr. No.	Mutant strain No.	Maximum cell mass produced (g/L)	Maximum lysine produced (g/L)	Total sugar consumed (g/L)	$Y_{p/x}$	$Y_{p/s}$	$Y_{x/s}$	$Y_{s/x}$
01	Parent strain	17.820	04.000	39.290	0.224	0.101	0.453	2.204
02	CGR-0l	16.042	10.920	38.470	0.681	0.284	0.417	2.399
03	CGH-02	19.504	15.530	39.520	0.796	0.393	0.494	2.026
04	CGR-03	18.070	12.710	39.026	0.703	0.326	0.463	2.160
05	CGR-04	19.000	14.970	39.947	0.788	0.375	0.476	2.102
06	CGR-05	18.990	13.020	39.512	0.686	0.329	0.481	2.08
07	CGR-06	16.529	10.870	37.230	0.658	0.292	0.444	2.252
08	CGR-07	19.982	16.770	39.821	0.842	0.421	0.502	1.992
09	CGR-08	20.521	19.510	39.990	0.951	0.488	0.513	1.949
10	CGH-09	20.978	20.040	40.075	0.550	0.500	0.523	1.910
11	CGH-10	17.032	11.580	38.709	0.680	0.299	0.440	2.272
12	CGH-11	19.750	17.720	39.537	0.897	0.448	0.500	2.001
13	CGH-12	16.680	10.850	37.861	0.650	0.287	0.440	2.269
14	CGH-13	16.042	9.3200	38.027	0.581	0.245	0.422	2.370
15	CGH-14	19.980	18.250	39.572	0.913	0.461	0.505	1.980
16	CGH-15	22.000	21. 792	40.063	0.990	0.544	0.549	1.821
17	CGH-16	19.721	15.600	39.241	0.790	0.398	0.503	1.990
18	CGH-17	17.532	11.650	37.870	0.664	0.305	0.463	2.160

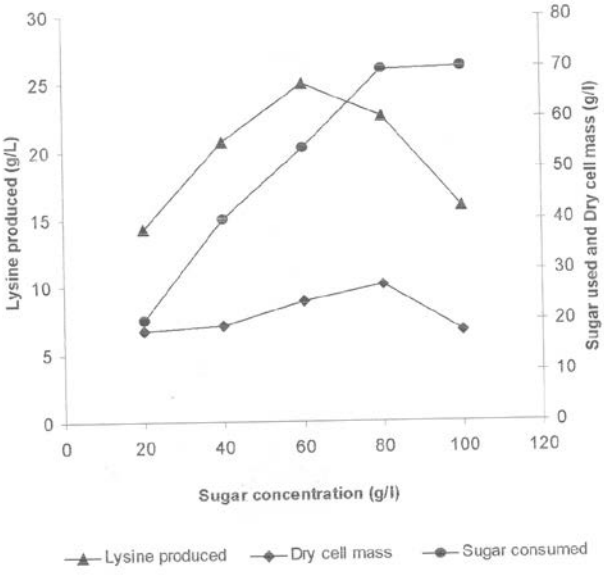

Fig. 1. Effect of different sugar concentrations on the production of lysine by CGH-15 mutant of the *C. glutamicum* in 2L fermenter in 72 hours.

Kinetic parameters for the parent and mutant strain

Different kinetic modals were developed for lysine production by *C. glutamicum* (Yang *et al.*, 1999; Hua *et al.*, 2000; Tada *et al.*, 2001; Ensari and Lim, 2003). Kinetic studies help us to bring in order

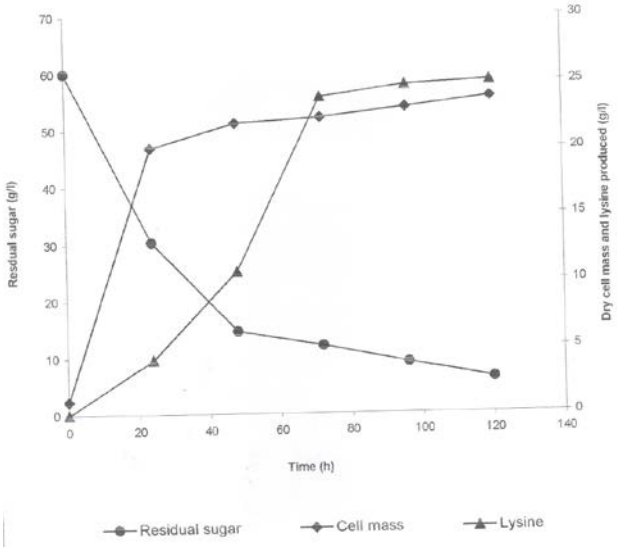

Fig. 2. Time profile of lysine fermentation by CGH-15 mutant of *Corynebacterium glutamicum* in 2L fermenter.

the mass of data which results from a practical fermentation experiment and to express the results in a concise form, which is intelligible to the colleagues and to those who make use of the results (Sinclair and Cantero, 1996).

Kinetic parameters were estimated for the homo serine auxotrophic mutant of *C. glutamicllm*, at 60, gl^{-1} initial sugar concentration, as it was found the most favorable concentration of initial sugar for lysine fermentation in a molasses based medium. Values of the estimated parameter's have been presented in Table II. Kinetic parameters of the parent and mutant strains were compared. Growth rate of the mutant strain was higher than the parent. This fact is also exhibited by the doubling time that was 2.84 hours for mutant and 3.22 hours for the parent. Volumetric rate of substrate utilization (Q_s) and specific rate of substrate utilization (q_s) had greater values for the parent than the mutant, which means that parent used more substrate as compared to the mutant. Product yield ($Y_{p/x}$) per gram of cells was increased many folds in case of mutant. Product yield ($Y_{p/s}$) too was higher for the mutant. Similar was the case for the volumetric rate of product formation (Q_p) and specific rate of product formation (q_p).

Tale II.- Comparison of kinetic parameters of the parent and mutant strain.

Sr. No.	Kinetic parameters	Parent strain	Mutant strain
For substrate consumption following growth			
1	μ (h)	0.221	0.257
2	Td (h)	3.22	2.840
3	Q_s (g/l.h)	1.640	1.238
4	Q_s (g/g.h)	0.570	0.38
For product formation following growth			
1	$Y_{p/x}$ (g.g)	2.124	35
2	$Y_{p/s}$ (g/g)	0.084	0.464
3	Q_p (g/l.h)	0.115	0.546
4	q_p (g/g.h)	0.457	8.54

Kinetic parameters: μ, Specific growth rate, Td; Doubling time, Q_s; Volumetric rate of substrate utilization, q_s; Specific rate of substrate utilization, Y pix; Product yield coefficient /g of cells, $Y_{p/s}$; Product yield coefficient /g of substrate, Q_p; Volumetric rate of product formation, q_p; Specific rate of product formation.

The mutant had higher growth rate and ultimately higher concentration of biomass and lysine as compared to the parent. Therefore, it can be concluded that the synthesis of lysine depends on both the growth rate and biomass concentration.

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