Pak. J. Bot., 44: 347-353, Special Issue March 2012

MUTAGENESIS INDUCED HYPERPRODUCTION OF L-LYSINE IN SHAKE FLASK AND FERMENTER BY *BREVIBACTERIUM FLAVUM* IIBUV2

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Abstract

Two bacterial cultures of *Corynebacterium glutamicum* and *Brevibacterium flavum* were screened for maximum L-lysine synthesis in shake flask. Among these, the best culture i.e., *B. flavum* was subjected to mutagenesis through UV irradiation, ethidium bromide and nitrous acid treatment for enhanced production of L-lysine. Among five isolated mutants screened for efficiency towards L-lysine synthesis *B. flavum* IIBUV2 was found to be the best producer. *B. flavum* IIBUV2 and its wild type were, then, compared to find out an appropriate cultivation media and conditions. Maximum L-lysine production of 8.8 g/l was obtained when mutant was cultivated on medium containing (%, w/v): glucose 3.0, peptone 0.5, NH_4SO_4 0.1 and K_2HPO_4 0.005, pH 7.5 at 32°C with 8% inoculum. When the mutant was cultivated in 7.5 L stirred fermenter the lysine yield was increase up to 17.8 g/l after 120h at 300 rpm agitation and 4.0 VVM aeration.

Introduction

Amino acids are biologically important compounds and basic units of proteins (Kinosionhita, 1959). There are twenty naturally existing amino acids and L-Lysine exists at the top of the list of nine essential and commercially important amino acids (Liebl *et al.,* 1991). L-lysine plays an important role in many biological processes; that is the reason why it is regarded as key element in health and nutrition of animals (Oh *et al.,* 1993). It also excites the cell division (Zelder *et al.,* 2005) and is necessary for the carnitine production, a component vital to convert fatty acids into simpler compounds and energy and facilitating the lowering of blood cholesterol, which is important for proper body functioning. L-lysine seams to assist the body take up and save calcium and it performs a critical role in collagen synthesis, an amino acid necessary for connective tissues and bones consisting of skin, tendon, and cartilage. The use of L-lysine can also prove helpful to overcome heart symptom like angina pectoris. L-lysine is an essential ingredient to clean arteries, which is very important for cancer prevention.

L-lysine producing cultures of the *Corynebacterium glutamicum*, *Brevibacterium flavum* and *B. lactofermentum* have been exploited to synthesize amino acids at industrial level since 1950s. Biosynthesis of L-amino acids has improved recently and is likely 2 million metric tons at present. Biosynthesis of L- amino acids is restricted to L-lysine and L-glutamic acid and few others at present (Zelder *et al.,* 2005). For cost effective production, improvement in fermentation technologies has become an essential industrial requirement. The synthetic distortions of metabolism can give the enhanced synthesis of specific amino acids (Nakayama, 1985). The most proficient mutation inducing chemicals are alkylating compounds, e.g., ethyl methane sulfonate (EMS), ethidium bromide, and the nitrous substances such as N-methyl-N-nitro-N-nitrosoguanidine (MNNG). Ultraviolet light treatment has been suggested as a mutagen of primary option. It can stimulate both frame shifts and base pair substitution (Bridge, 1976). For the improvements in fermentation processes, researches are constantly being conducted in the field of biochemical engineering including media optimization (e.g., glucose concentration, additives and essential nutrients additives) as well as strain and process improvement (e.g., agitation, aeration, pH, carbon dioxide and temperature). Various optimum and operational temperatures have been used for L-lysine synthesis. Physical conditions (agitation, inoculum density, inoculum age, pH and temperature) and medium composition and incubation time very powerfully enhance the L-lysine synthesis. Mutants make possible the economical manufacturing of amino acids by utilizing inexpensive carbon supply (Wang *et al*., 1994). The L-lysine requirement is gradually increasing in the field of feeds, foods, pharmaceuticals and chemicals, etc. To fulfill rising and varied L-lysine requirements, there is still scope for enhancement in stains based on the understanding of microbial metabolism. Moreover research on method optimization, particularly for lessening the expenditure of carbon and energy source is also beneficial.

Present study for L-lysine production was carried out to increase the bioreactor productivity by systematic media optimization, strain improvement and adaptation of fermentation conditions for the development of the process.

Materials and Method

Three *Corynebacterium glutamicum* stains and one *Brevibacterium flavum* strain were obtained from stock cultures of Pakistan Council of Scientific and Industrial Research, Lahore and University of Health Sciences, Lahore. Medium for inoculums preparation included peptone (1% w/v), yeast extract 1.5 (%w/v), NaCl (0.5% w/v), pH 7.0. Fifty ml of the medium taken in conical flasks was subjected to sterilization by autoclaving at 121°C for 15 minutes. The flasks were inoculated with 2.0-4.0% microorganisms and incubated on a shaking incubator at 200 rpm and 30°C.

Medium utilized for the fermentation purposes consisted of glucose 30 g/l, $(NH_4)_2SO_4$ 20 g/l, Urea 3 g/l, MgSO4. $7H_2O$ 0.5 g/l, Soy hydrolysate 20 g/l, KH_2PO_4 1.0 g/l, $MnSO_4$. $4H_2O$ 0.002 g/l, Biotin 0.0001 g/l, Thiamin HCl 0.0001 g/l (Oh *et al*., 1993). Fermentation medium (50 ml) was taken in a 250 ml conical flask. After sterilization, it was inoculated and incubated on rotary shaking incubator with 200 rpm at 30±2°C for 120 h. After completion of incubation period, the fermented broth was subjected to centrifugation at 6,000 rpm for 10 minutes and supernatant of the fermented broth was used for analytical purposes.

For the sake of culture improvement, the screened out L-lysine producing *B. flavum* was treated with various mutagens. For UV mutagenesis the parental bacterial culture, 1.00 ml inoculum suspension about 18 h old was transferred to a sterilized petriplates containing NB medium and these cells were subjected to ultraviolet irradiation (UV irradiation) exposure for 90 minutes under the beam (λ=253 nm and 220 V at 50 c/s) of UV lamp (Model: Mineral Light, UVS-12, California, USA) with 15 minutes time interval. A distance of 8.0 cm was adjusted between lamp and cell suspension for each trial to obtain more than 95% death rate (Azin & Noroozi, 2001).

For chemical mutation the cells of parent strain, *Brevibacterium flavum* grown on nutrient broth medium with an approximate population density of 3 x 10^8 cells per ml were subjected towards mutagens. Each colony, appeared, was picked with sterile toothpick and plated.

Scale up studies was conducted in a 7.5 L fermenter (Brunswick, USA). Fermentation process was carried out at 200-300 rpm, pH 7.5, dissolved oxygen (DO) 2-4 vvm, inoculum size 8% for 120 hours at 30±2°C.

The samples for analysis were taken at different time intervals and fermentation broth. The supernatant obtained after centrifugation was used for estimation of biomass, residual glucose and for Lysine yield. The cell weight was determined by drying at 105°C overnight in an oven. DNS (Dinitro salicylic acid) method was employed to estimate the residual glucose (Tasun *et al.,* 1970). Ninhydrin-Ferric reagent method was followed for the estimation of L-lysine (Chung- Lung *et al.,* 1995). The transmittance was measured at 470 nm by using scanning spectrophotometer (Hitachi, U-2000, Japan).

For processing the data, statistical methods given by Snedecor & Cochran (1980) was adopted by using Costat Computer software.

Results and Discussion

Screening of wild strains revealed that all the strains of *Corynebacterium glutamicum* and *Brevebacterium flavum* accumulated L-lysine in the culture broth. However only *B. flavum* revealed efficient productive ability to be considered for commercial utilization. It was observed that wild strains isolated from nature could not give appreciable amounts of L-lysine. The main cause of this fact was the cellular regulation, the impact of which has been well documented. Thus efforts were made to induce random mutation. The aim of using mutants was to get rid off feed back control mechanisms through limiting the accumulation of feedback suppressing substances e.g., successful L-lysine fermentation was designed with homoserine dependant mutants (Nakayama *et al*., 1961).In the current study, the lysine productivity was enhanced through exposure to ultraviolet (UV) light, which made possible the bacteria to display an improvement in L-lysine production. During mutation studies by using UV light treatment two *Brevibacterium flavum* mutants (*B. flavum* IIBUV1 and *B. flavum* IIBUV2) were isolated which were able to produce 6 g/l and 9 g/l of lysine respectively after 120 hours of fermentation in shaking incubator In Ethidium bromide treated cultures showed L-lysine production potential of 6.23 and 3.76 for *B. flavum* EtBr1 and *B. flavum* EtBr2 respectively. Nitrous acid induced mutant revealed relatively lesser yield in comparison with other mutants. *B. flavum* IIBUV2 gave utmost lysine production after 120 h of growth and was selected to be considered for further studies. Experiments with mutant strains exposed differences in production characteristics, from the point of view of consumption of total sugar during synthesis, amount of lysine formed and concentration of biomass at the stop of fermentation (Table 1).

Table 1. Screening of *Brevibacterium flavum* mutants for the production of L-lysine in shake flask.

Mutants	L-lysine (g/l)	Residual glucose (g/l)	Biomass (g/l)
B. flavum IIBUV1	7.4 ± 0.02 [b]	0.43 ± 0.1[d]	8.6 ± 0.2 [b]
B. flavum IIBUV2	9.01 ± 0.01 [a]	0.38 ± 0.015 [e]	10.3 ± 0.15 [a]
B. flavum IIBEtBr1	6.23 ± 0.02 [c]	0.48 ± 0.01 [c]	8.0 ± 0.12 [c]
B. flavum IIBEtBr2	3.76 ± 0.01 [e]	0.7 ± 0.02 [a]	7.2 ± 0.09 [d]
B. flavum IIBHN1	5.9 ± 0.015 [d]	0.64 ± 0.019 [b]	6.01 ± 0.2 [e]
LSD	0.02	0.007	0.068

Each value is an average of three replicates, ± denoted standard deviation among replicates, numbers followed by different letters differs significantly at ∝ =0.05, Temperature 30°C, pH and Agitation 200 rpm

The impact of sucrose, fructose, molasses and starch on biomass and L-lysine synthesis was evaluated in comparison with that of glucose (Table 2). It emerged that both sucrose and glucose boost lysine synthesis. Sucrose was the second best carbon source for L-lysine production (6.8 g/l) and biomass production (8.02 g/l). Ferreria and Durate (1991) utilized glucose for the highest yield of L-lysine by *Corynebacterium glutamicum*. Hadj-Sassi *et al*., (1988) calculated that primary concentration of glucose enhanced the synthesis of L-lysine in batch culture and observed that particular synthesis rate was found at 65 g/l of glucose. Biosynthesis of lysine was declined to 3.7 g/l and 1.09 g/l respectively, when molasses were added to the medium. The yield of L-lysine was low and the utilization of carbon sources was also somewhat lower in case of molasses.

Different ammonium salts (Table 3) e.g., ammonium sulphate, ammonium chloride, ammonium nitrate and urea can be easily absorbed by the various microorganisms. Among various nitrogen sources considered, it was found that ammonium sulphate is the finest one for the synthesis of L-lysine. L-lysine yield obtained by using ammonium sulphate (7.43 g/l) as nitrogen source was significantly greater than urea (4.67 g/l), ammonium chloride (4.82 g/l) and ammonium nitrate (3.34 g/l). This is in accord with Plachy (1969) using *Corynebacterium sp.* 9366 h454 for lysine production by utilizing ammonium sulphate as nitrogen source. Highest L-lysine built up (7.43 g/l) was observed at 2% ammonium sulphate concentrations. Wang *et al*., (1994) recommended more than 2% ammonium sulphate for L-lysine proved unfavorable for biomass. Ammonium sulphate was utilized the nitrogen source for L-lysine fermentation by many researchers (Zaki *et al.,* 1982; Ferreria & Durate, 1991).

Table 2. Screening of different carbon sources on L-lysine production from *B. flavum* mutant and its wild type in shake flasks.

Source of carbon ammonium sulphate concentrations % w/v	Mutant			Wild		
	L-lysine (g/l)	Residual sugar (g/l)	Biomass (g/l)	L-lysine (g/l)	Residual sugar (g/l)	Biomass (g/l)
Lactose	1.63 ± 0.02 [f]	0.5 ± 0.01 [c]	4.97 ± 0.1 [e]	1.18 ± 0.002 [d]	1 ± 0.004 [d]	5.4 ± 0.04 [c]
Glucose	7.55 ± 0.1 [a]	0.04 ± 0.00 [c]	10.03 ± 0.2 [a]	4.65 ± 0.09 [a]	0.79 ± 0.005 [de]	6.12± 0.01 [b]
Sucrose	6.8 ± 0.15 [b]	0.09 ± 0.002 [c]	8.13 ± 0.15 [b]	2.41 ± 0.01 [b]	0.43 ± 0.01 [e]	7.9 ± 0.2 [a]
Fructose	2.92 ± 0.05 [e]	3.66 ± 0.01 [a]	7.16 ± 0.1 [c]	1.1 ± 0.001 [d]	2.5 ± 0.1 [c]	6.18 ± 0.08 [b]
Maltose	4.5 ± 0.03 [c]	3.1 ± 0.02 [b]	8.09 ± 0.12 [b]	1.89 ± 0.004 [c]	3.6 ± 0.03 [b]	4.54 ± 0.015 [d]
Molasses	3.77 ± 0.01 [d]	3.30 ± 0.015 [ab]	5.87 ± 0.09 [d]	1.09± 0.002 [d]	6.9 ± 0.023 [a]	4.23 ± 0.05 [d]
LSD	0.159	0.478	0.077	0.148	0.4	0.39

Each value is an average of three replicates, ± denoted standard deviation among replicates, numbers followed by different letters differs significantly at ∝ =0.05, Temperature 30°C, pH 7.00 and Agitation 200 rpm

Table 3. Effect of different nitrogen sources on L-lysine production from *B. flavum* mutant and its wild type in shake flasks.

Source of nitrogen ammonium sulphate concentrations % w/v	Mutant			Wild		
	L-lysine (g/l)	Residual glucose (g/l)	Biomass (g/l)	L-lysine (g/l)	Residual sugar (g/l)	Biomass (g/l)
Ammonium sulphate	7.43 ± 0.1 [a]	0.03 ± 0.05 [a]	7.1 ± 0.1 [a]	2.44 ± 0.1 [a]	0.04± 0.03 [b]	5.66 ± 0.11 [a]
Urea	4.67 ± 0.13 [b]	0.07 ± 0.02 [a]	5.01± 0.16 [c]	2.13 ± 0.14 [b]	0.1 ± 0.04 [b]	4.19 ± 0.06 [c]
Ammonium Chloride	4.82 ± 0.16 [b]	0.05 ± 0.02 [a]	6.02 ± 0.06 [b]	2.09± 0.07 [b]	0.6 ± 0.01 [a]	5.1 ± 0.05 [b]
Ammonium nitrate	3.34 ± 0.2 [c]	0.06 ± 0.003 [a]	4.43 ± 0.3 [d]	1.73 ± 0.11 [c]	0.1 ± 0.03 [b]	4.02 ± 0.1 [c]
LSD	0.3	0.07	0.29	0.041	0.6	0.13

Each value is an average of three replicates, ± denoted standard deviation among replicates, numbers followed by different letters differs significantly at ∝ =0.05, Temperature 30°C, pH 7.00 and Agitation 200 rpm

Biotin is mandatory for the appropriate synthesis of L-lysine. Young and Chipely 1984 explored the outcome of biotin on L-lysine fermentation in the *Brevibacterium lactofermentum*, and it was observed that biotin treated cell built up more glucose than the untreated one. Biotin actually induced a few modifications in the composition of cell wall, permitting an enhancement in accumulation of sugar. The uptake studies reveal that biotin affects the cell surface probably the bacterial membrane. It is well known that bacterial membrane plays an important role as charged barrier. This mechanism seems to be responsible for regulation of amount of L-lysine excreted by the bacterial cells. Tosaka *et al.,* (1986) suggested this effect might be due to activation of pyruvate carboxylase by biotin. In the current studies the effect of concentration of biotin (50-300 μg/l) was examined. An enhancement in the biomass production was observed with increase in the biotin concentration up to 200 μg/l of biotin and beyond this concentration biomass production became constant. The highest L-lysine synthesis (7.93 g/l) and biomass production (7.9 g/l) was gained with the 200 μg/l of biotin in case of *B. flavum* IIBUV2 (Table 4).

Table 4. Effect of different concentrations of biotin on L-lysine production from *B. flavum* mutant and its wild type in shake flasks.

Biotin concentration (ug/l) ammonium sulphate concentrations % w/v	Mutant			Wild		
	L-lysine (g/l)	Residual sugar (g/l)	Biomass (g/l)	L-lysine (g/l)	Residual sugar (g/l)	Biomass (g/l)
50	5.04 ± 0.12 [d]	0.66 ± 0.09 [a]	4.9 ± 0.09 [d]	3.0 [d] ± 0.1	1.8 ± 0.1 [a]	3.5 ± 0.1 [d]
100	5.9 ± 0.2 [c]	0.53 ± 0.05 [a]	5.3 ± 0.1 [c]	3.3 ± 0.3 [c]	1.5 ± 0.3 [a]	4.01 ± 0.5 [c]
150	6.8 ± 0.1 [b]	0.3 ± 0.07 [b]	6.1± 0.11 [b]	4.02 ± 0.24 [b]	1.0 ± 0.1 [b]	4.7 ± 0.22 [b]
200	7.93 ± 0.15 [a]	0.1 ± 0.06 [c]	7.9 ± 0.1 [a]	4.71 ± 0.2 [a]	0.3 ± 0.05 [c]	5.2 ± 0.2 [a]
250	7.7 ± 0.17 [a]	0.14 ± 0.01 [bc]	7.8 ± 0.15 [a]	4.65 ± 0.1 [a]	0.31± 0.08 [c]	5.1 ± 0.1 [ab]
LSD	0.229	0.16	0.2	0.296	0.318	0.409

Each value is an average of three replicates, ± denoted standard deviation among replicates, numbers followed by different letters differs significantly at ∝ =0.05, Temperature 30°C, pH 7.00 and Agitation 200 rpm

Enfluence of inoculum mass on the L-lysine synthesis was experienced. Low mass of inoculum may give an insufficient biomass formation and too high mass may confer too much biomass and exhaust the substrate of substances crucial for L-lysine synthesis. Inoculum density from 2-10% was calculated on shake flask and biomass production and L-lysine synthesis increased subsequently. The best results were acquired at 8.0%

inoculum density. The left over sugar reduced to 0.1 g/l and utmost L-lysine production experienced was 7.8 g/l by mutant strain. Beyond 8.0% inoculum size, a slight decline in the L-lysine synthesis and biomass production was observed. Residual sugar was also significantly greater with 10.0% inoculum size both in case of mutant and parent strain. Undersized inoculum resulted an enhancement in growth phase, too low mass may provide inadequate cell biomass. It may not be appropriate to state that 8% inoculum volume in all fermentation processes, as it relies on biomass and the composition of the start medium to be conveyed.

Rate of L-lysine buildup was explored by shake flask experimentations (Table 5). After 24 h, 2.7 g/l of l-lysine was estimated, while upto 40% of the sugar was utilized. A sharp increase in the L-lysine synthesis, biomass production and glucose consumption was seen in the initial 96-120 h hours after that the rate of biomass production and L-lysine synthesis started to become constant. This decline occurred probably due to diminishing of nutrients and feed back inhibition effects. A lysine yield of 7.8 g/l was obtained and the remaining glucose was about 1.0% after 96 h fermentation.

Table 5. Rate of L-lysine production from *B. flavum* mutant and its wild type in shake flasks.

Time (hours) ammonium sulphate concentrations % w/v	Mutant			Wild		
	L-lysine (g/l)	Residual sugar (g/l)	Biomass (g/l)	L-lysine (g/l)	Residual sugar (g/l)	Biomass (g/l)
24	2.7 ± 0.05^{e}	11.0 ± 0.4^{a}	4.0 ± 0.1^{e}	1.83 ± 0.09^{f}	6.0 ± 0.5^{a}	3.02 ± 0.05^{f}
48	4.29 ± 0.1^{d}	7.0 ± 0.1^{b}	6.8 ± 0.18^{d}	2.5 ± 0.2^{e}	5.1 ± 0.2^{b}	3.45 ± 0.05^{e}
72	6.31 ± 0.3^{c}	3.5 ± 0.05^{c}	7.5 ± 0.2^{c}	3.32 ± 0.1^{d}	3 ± 0.1^{c}	4.8 ± 0.01^{d}
96	7.89 ± 0.2^{b}	0.72 ± 0.01^{d}	9.6 ± 0.2^{b}	3.97 ± 0.2^{c}	2.1 ± 0.05^{d}	5.5 ± 0.2^{c}
120	8.31 ± 0.2^{a}	0.046 ± 0.003^{e}	11.63 ± 0.5^{a}	4.19 ± 0.11^{b}	1.9 ± 0.1^{e}	5.5 ± 0.3^{b}
144	8.3 ± 0.11^{a}	0.046 ± 0.005^{e}	11.6 ± 0.2^{a}	4.56 ± 0.16^{a}	1.8 ± 0.2^{e}	6.1 ± 0.25^{a}
168	8.3 ± 0.18^{a}	0.046 ± 0.002^{e}	11.6 ± 0.3^{a}	4.56 ± 0.2^{a}	1.8 ± 0.1^{e}	6.1 ± 0.15^{a}
LSD	0.29	0.147	0.124	0.1	0.15	0.13

Each value is an average of three replicates, ± denoted standard deviation among replicates, numbers followed by different letters differs significantly at $\propto$ =0.05, Temperature 30°C, pH 7.00 and Agitation 200 rpm

The outcome of different pH on L-lysine production was studied (Table 6). Kikuchi *et al.,* (1999) described initial pH of culture medium between 6.0 and 8.0 for L-lysine fermentation. In the present studies, there was no growth observed at pH 5.0, however, there was little growth at pH 5.5. Both biomass production (7.4 g/l) and l-lysine synthesis (6.6 g/l) obtained at pH 7.0, were significantly greater than the results obtained at pH 6.0 and 6.5. This result is in accordance with studies conducted by Nakamura *et al.,* (2000), who described a pH in the range of 5.8 to 8.5, preferably 6.5 to 7.5 for L-lysine synthesis using *Corynebacteria*, whereas L-lysine production was obtained at pH 7.2 by Stevens & Binder (2004) and Liaw *et al*., (2006). Maximum L-lysine synthesis (8.4 g/l) was experienced at pH 7.5. Maximum biomass production (10.0 g/l) was also observed at pH 7.5. Biomass growth and L-lysine production almost completely ceased beyond pH 8.5. Liu *et al.,* (1986) accounted that L-lysine synthesis dropped off at pH lower than 6.5 but observed no differentiation between 6.5-8.0. An initial pH of 7.5 was recommended for L-lysine synthesis by *Corynebacterium glutamicum* (Boer & Kramer, 1991). Boer *et al.,* (1993) indicated that optimal pH for L-lysine synthesis was 7.4-7.8.

Table 6. Effect of pH on L-lysine production from *B. flavum* mutant and its wild type in shake flasks.

pH	Mutant			Wild		
	L-lysine (g/l)	Residual sugar (g/l)	Biomass (g/l)	L-lysine (g/l)	Residual sugar (g/l)	Biomass (g/l)
5.00	0.0 ± 0.0^{i}	25 ± 0.0^{a}	0.0 ± 0.0^{i}	0.0 ± 0.0^{h}	25.0 ± 0.0^{a}	0.0 ± 0.0^{i}
5.50	0.5 ± 0.02^{h}	22 ± 0.5^{b}	0.84 ± 0.1^{h}	0.00 ± 0.0^{h}	24.0 ± 0.02^{b}	0.813 ± 0.005^{h}
6.00	2.72 ± 0.1^{f}	15 ± 0.3^{c}	3.65 ± 0.21^{f}	0.9 ± 0.05^{g}	19.1 ± 0.5^{c}	2.3 ± 0.15^{g}
6.50	4.13 ± 0.16^{d}	10 ± 0.1^{d}	5.91 ± 0.25^{d}	2.11 ± 0.1^{e}	12.2 ± 0.3^{d}	4.4 ± 0.2^{d}
7.00	6.65 ± 0.15^{b}	3 ± 0.12^{g}	7.45 ± 0.16^{c}	4.35 ± 0.2^{b}	4.1 ± 0.1^{h}	5.98 ± 0.3^{b}
7.50	8.42 ± 0.3^{a}	0.3 ± 0.03^{h}	10.34 ± 0.2^{a}	4.98 ± 0.17^{a}	2.1 ± 0.05^{h}	6.32 ± 0.17^{a}
8.00	5.9 ± 0.2^{c}	3.0 ± 0.01^{g}	8.01 ± 0.3^{b}	3.78 ± 0.05^{c}	2.7 ± 0.2^{g}	5.81 ± 0.09^{c}
8.50	3.23 ± 0.1^{e}	4.1 ± 0.03^{f}	5.43 ± 0.1^{e}	2.51 ± 0.2^{d}	4.1 ± 0.12^{f}	3.5 ± 0.19^{e}
9.00	2.1 ± 0.11^{g}	5.0 ± 0.05^{e}	3.02 ± 0.05^{g}	1.28 ± 0.01^{f}	7.6 ± 0.3^{e}	2.2 ± 0.1^{f}
LSD	0.153	0.5	0.23	0.116	0.5	0.13

Each value is an average of three replicates, ± denoted standard deviation among replicates, numbers followed by different letters differs significantly at $\propto$ =0.05,Temperature 30°C, Time 120 hours and Agitation 200 rpm

It must be understood that the changes in temperature can alter the utilization rate of one constituent as compared to another, thus unsettling the equilibrium of the medium with respect to the biomass growth. So temperature control is indispensable for acquiring reproducible results. The influence of different temperatures on the L-lysine synthesis was considered on shaking incubater (Table 7). The highest L-lysine yield (8.8 g/l) and biomass production (9.3 g/l) was obtained at 32°C . Liaw *et al.,* (2006) and Broer *et al.*, (1991) operated at about 32°C for l-lysine synthesis by using *Corynebacteria.* Temperature above 32°C appreciably reduced the L-lysine synthesis (Hilliger *et al.,* 1984).

Oxygen is an indispensable element for L-lysine fermentation. Under unsatisfactory oxygen circumstances bulk quantity of succinic and Lactic acids build up, whereas surplus oxygen enhances the quantity of alpha-keto glutaric acid. Plentiful oxygen supply and short oxygen availibity are undesireable. The former being restraining to biomass and latter to L-lysine synthesis (Liu, 1986). The utmost L-lysine synthesis (15.5 g/l) and sugar utilization were observed at 4 vvm of oxygen (Fig. 1). This is in accordance with studies of Plachy *et al.,* (1969) L-lysine synthesis and sugar utilization diminished with the subsequent reduction in oxygen supply. The highest biomass yield was also observed at 4 vvm of oxygen supply. Hadj-sassi *et al.,* (1996) investigaated that insufficient O2 is responsible for reduction in utilization of substrate and conversion effectiveness of substrate into L-lysine.

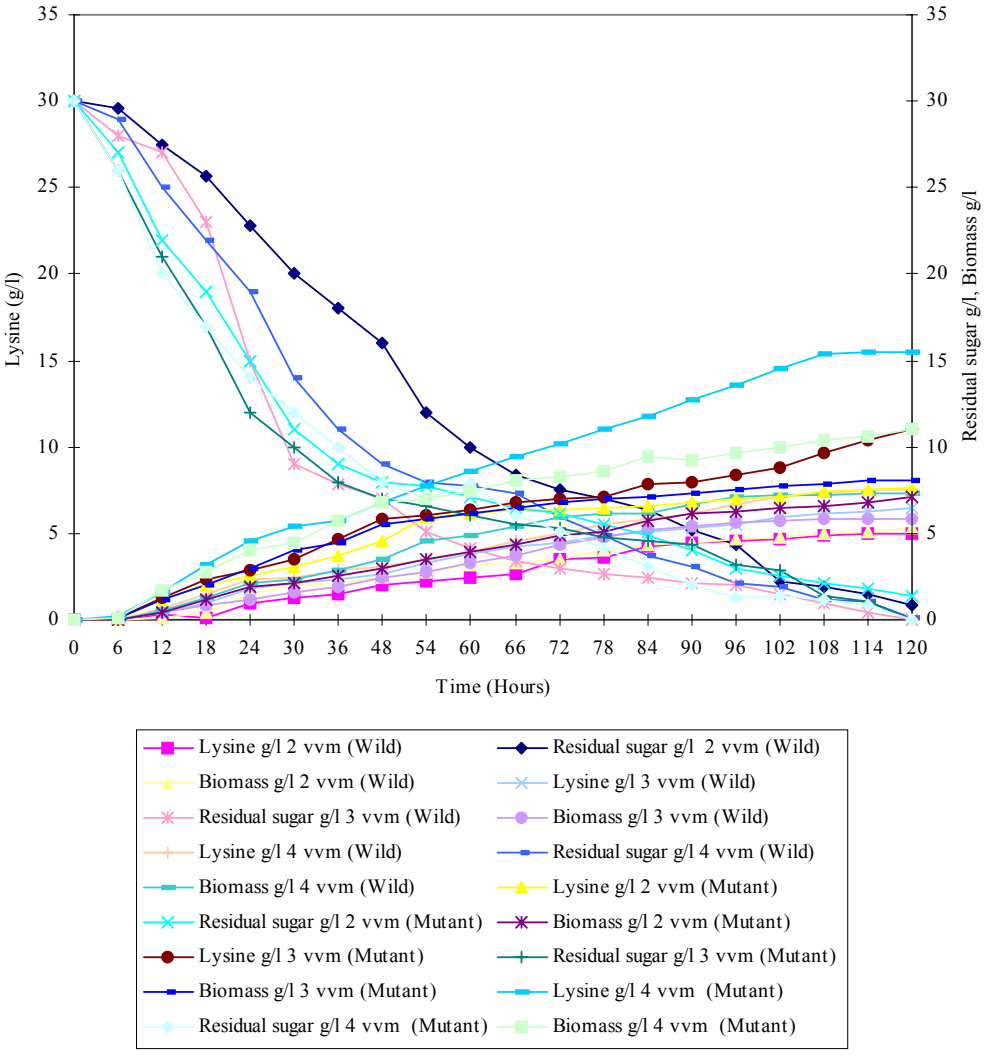

Fig. 1. Effect of aeration on L-lysine production from *B. flavum* mutant and its wild type in 7.5 l fermenter (Temperature 30-32°C, pH 7.50 and agitation 200 rpm).

Table 7. Effect of incubation temperature for L-lysine production from *B. flavum* mutant and its wild type in shake flasks.

Temperature (°C)	Mutant			Wild		
	L-lysine (g/l)	Residual sugar (g/l)	Biomass (g/l)	L-lysine (g/l)	Residual sugar (g/l)	Biomass (g/l)
28	5.57 ± 0.1 [e]	2.5 ± 0.11 [a]	6.5± 0.1 [e]	3.57 ± 0.2 [de]	4.6 ± 0.3 [bc]	3.64 ± 0.05 [f]
29	6.22 ± 0.2 [d]	2 ± 0.2 [b]	6.65 ± 0.3 [e]	4.32 ± 0.1 [c]	4.0 ± 0.1 [de]	4.73 ± 0.1 [d]
30	7.45 ± 0.15 [c]	1.3 ± 0.2 [c]	7.8 ± 0.14 [d]	4.85 [a] ± 0.05	1.4 ± 0.12 [h]	6.73 ± 0.07 [a]
31	8.15 ± 0.21 [b]	1 ± 0.2 [cd]	8.86 ± 0.15 [b]	4.55 ± 0.05 [b]	2.4 ± 0.1 [g]	6.6 ± 0.1 [a]
32	8.8 ± 0.2 [a]	0.4 ± 0.09 [e]	9.39 ± 0.265 [a]	4.2 ± 0.15 [c]	2.9 ± 0.27 [f]	6.05± 0.15 [b]
33	7.74 ± 0.15 [c]	0.73 ± 0.2 [de]	8.33± 0.149 [c]	3.74 ± 0.2 [d]	3.6 ± 0.3 [e]	5.11± 0.2 [c]
34	5.69 ± 0.1 [e]	1.9 ± 0.1 [b]	6.2 ± 0.1 [f]	3.69 ± 0.3 [d]	4.3 ± 0.3 [cd]	5.0 ± 0.2 [cd]
35	5.23 ± 0.21 [f]	2.5 ± 0.3 [a]	5.74 ± 0.16 [g]	3.33 ± 0.28 [e]	5.0 ± 0.29 [b]	4.42 ± 0.31 [e]
36	4.47 ± 0.18 [g]	2.8 ± 0.25 [a]	5.53 ± 0.2 [g]	2.97 ± 0.1 [f]	6.01 ± 0.5 [a]	3.2 ± 0.15 [g]
L.S.D.	0.25	0.443	0.24	0.49	0.288	0.37

Each value is an average of three replicates, ± denoted standard deviation among replicates, numbers followed by different letters differs significantly at $\propto$ =0.05, Time 120 hours, pH 7.00 and Agitation 200 rpm

Fermenter vessel containing liquid medium and inoculum was agitated to give uniform mixing. The outcome of stirring on L-lysine synthesis in the 7.5 L fermenter was examined. In the fermenter, as the agitation increased from 100-300 rpm, L-lysine production improved quickly (Fig. 2). Kreutzer *et al*., (2001) and Bathe *et al*., (2004) described the agitation rate of 250 to 300 rpm for the production of L-lysine in stirred fermenter. The highest L-lysine yield observed was 17.5 g/l at 300 rpm after 114 hours of fermentation. Oh *et al*., (1993) and Liaw *et al*., (2006) describes bench scale fermentations operating at 2.1 vvm aeration rate of atmospheric air and changing agitation speed during the fermentation between 600 and 900 rpm.

Fig. 2. Effect of agiation on L-lysine production from *B. flavum* mutant and its wild type in 7.5 l fermenter (Temperature 30-32°C, pH 7.50 and aeration 4.0 vvm).

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(Received for publication 12 February 2011)