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*Full Length Research Paper*

# Optimization and hyper-expressed production of lysine through chemical mutagenesis of *Brevibacterium flavum* by N-nitroso-N-ethylurea

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**One of best-known commercially produced amino acids is lysine, used as a feed additive to enhance its nutritional value. Some microorganisms have the capability of producing high amounts of lysine. *Brevibacterium flavum* was used for lysine production in this study. The bacterium was grown on nutrient agar medium. Following the optimization of culture conditions, the maximum growth was achieved after incubating the bacterial cells at 30°C (at pH 7.0), in the presence of 20 g/l corn steep liquor and glucose each, for 24 h. Incorporation of lactate, in the range between 2-12%, increased lysine production significantly (P>0.05). The mutagen, N-nitroso-N-ethylurea (ENU) was used for the hyper-production of lysine. Before initiating the mutation program, *B. flavum* was grown on suitable production medium. Cells were then exposed to 35 mM ENU for 5-30 min. After washing the bacterial pellet with sodium citrate buffer and growing on seed culture medium containing methionine and threonine as auxotrophic requirements, lysine production was enhanced up to 125 g/l in the culture exposed to the mutagen for 5 min as compared to 81 g/l, produced from wild type bacterial cells (P>0.001). These findings indicate the increased lysine production capacity of *B. flavum* in the presence of appropriate amounts of essential nutrients, added to the culture medium.**

**Key words:**Auxotrophs, *Brevibacterium flavum*, lysine, N-nitroso-N-ethylura, mutants.

## INTRODUCTION

Lysine is an important amino acid in animal nutrition (Jose et al., 2005). It is used as a feed additive to enhance their nutritional value. Poultry and livestock are unable to synthesize this amino acid. So, it must be added to their feedstuffs to provide an adequate diet. It is argued that the use of synthetic amino acids could reduce nitrogen pollution from animal wastes, as a result of better and more efficient nutrient utilization (Chadd, 2002). Auxotrophic and regulatory mutants of *Brevibacterium* species can produce lysine in substantial amounts (Paulus, 1993; Ali et al., 2009). During lysine production, threonine and methionine supply should be carefully regulated.

It is reported that lysine productivity increased when threonine feeding was regulated (Kiss and Stephanopoulos, 1991; Ali et al., 2009). This production occurs with classically obtained mutants derived by many rounds of undirected mutagenesis and screening. As it lacks lysine decarboxylase activity, it cannot degrade lysine. Production depends on how lysine is used as a nutrient. Lysine, used by humans, is ten times more expensive than that used by animals because a more highly refined product is needed for human use (Crouger and Annelise, 1990; Jakobsen et al., 2009). Mutation approach has become the most extensively used tool for industrial organisms (Lee et al., 1996). Chemical

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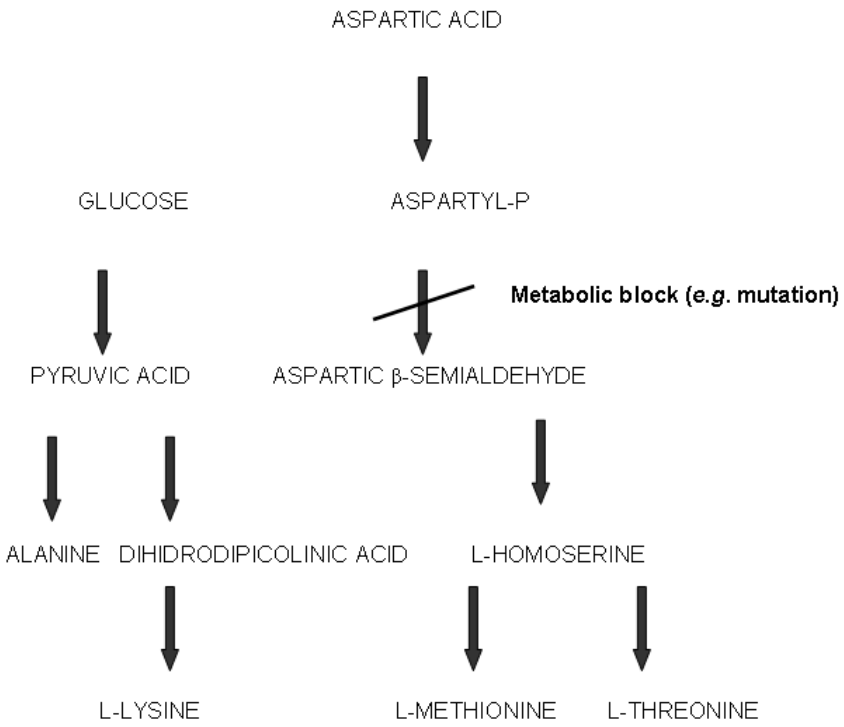


**Figure 1.** Schematic diagram of lysine production metabolism from L-homoserine.

mutagens are employed to the cell to an adjusted level so that 90-99% of the population is killed. This kill is caused by some lethal mutations that prevent the formation of critical components of the cell (Schendel and Flickinger, 1996). N-nitroso-N-ethylurea is a potent mutagen and carcinogen, now widely used by bacterial geneticists because it induces a high frequency of mutations, mostly at the replication fork with primary base substitutions, although small deletions have also been seen. The mutagenic power of N-nitroso-N-ethylurea (ENU) stems from the generation of diazomethane (Ohnishi et al., 2005; Wittmann and Hinzle, 2002). Almost one out of three hundred survivors has a mutation somewhere in *lac* operon. In fact, the replication region is 200 times more susceptible to mutation by ENU than the remainder of chromosome (Wittmann and Hinzle, 2002).

Considering the insufficient food supply worldwide and the ability of *Brevibacterium flavum* to produce essential amino acids like lysine, this bacterial strain would be an ideal source of getting the essential supplements as compared to conventional protein sources. Here we report, for the first time, the successful construction of auxotrophic mutants of *B. flavum* through the chemical mutagenesis by using ENU.

## MATERIALS AND METHODS

### Bacterial strain

The strain utilized in the present study, *B. flavum* 13826, was purchased from American Type Culture Collection (ATCC). The major product of this bacterial strain is lysine but this production can be enhanced by chemical mutagenesis. Lysine production metabolism involves TCA (tricarboxylic acid) cycle. Aspartate, synthesized from oxaloacetate by aspartate aminotransferase (glutamate:oxaloacetate transaminase), is converted into aspartic-β-semialdehyde, which yields homoserine and ε-diaminopimelate. Homoserine is further converted into L-isomers of threonine and methionine but ε-diaminopimelate produces lysine. In this branched pathway, chemical mutation blocks the pathway leading to the production of methionine and threonine (Figure 1). Absence of these metabolites in the growth medium causes decreased lysine production. In order to overproduce lysine, we need to avoid the inhibition by using an auxotrophic mutant of threonine and methionine.

### Bacterial cell cultures

The pre-cultures were grown in complex medium of glucose broth or Nutrient broth (3.75 g/l yeast extract, 10 g/l glucose, 5 g/l NaCl, 5 g/l peptone, 25 g/l $KH_2PO_4$, 25 g/l $MgSO_4$, 1 g/l $FeSO_4$, 0.5 g/l $ZnSO_4$, 0.5 g/l $MnSO_4$, 25 g/l $K_2HPO_4$) at 30°C with aeration. Culture conditions were optimized to get optimum levels of substrate, pH, temperature, incubation time and the growth enhancer (lactate). For agar plates, the complex medium was additionally amended with 25 g/l of agar. For the production of cells as an inoculum, the glucose broth was used without any additional nutrient. pH of the medium was adjusted to 7.0. The culture was incubated in orbital shaker (set at 150 rpm) for 24 h.

### Mutagenesis

Seed culture medium (D-glucose 10 g/l, peptone 5 g/l, yeast extract 3.75 g/l, NaCl 5 g/l, $(NH_4)_2SO_4$ 17.5 g/l, $K_2HPO_4$ 25 g/l, $KH_2P0_4$ 25

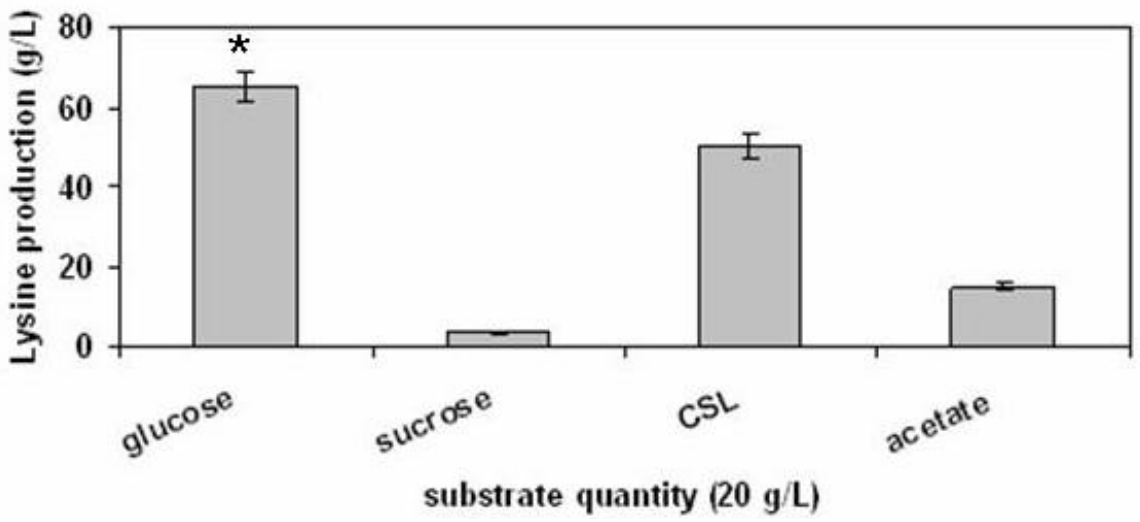


**Figure 2.** Effect of different substrates on lysine production from *B. flavum*. Effect of 20 g/l of (A) glucose, (B) sucrose, (C) corn steep liquor (CSL) and (D) acetate was seen on lysine production capacity of *B. flavum*. The cultures were grown separately with all the substrates and after spectrophotometric study; the results were compared with each other to find out the substrate, which can give maximum lysine production on optimum conditions of other parameters (* shows significant results and P>0.05 was considered to be significant).

g/l, threonine 20 g/l, methionine 20 g/l, $ZnSO_4$ 0.5 g/l; $MgSO_4.7H_20$ 25 g/l; $FeSO_4$. $7H_20$ 1 g/l, $MnSO_4$. $5H_20$ 0.5 g/l) was made for the growth of mutant bacterial cells. Wild type bacterial cells were grown on nutrient broth and 3 ml of this growth was inoculated in each of the 8 centrifuge tubes used for mutagenesis purpose. The cultures were incubated at 30°C for 24 h in orbital shaker. Cells were pelleted by centrifugation at 10,000×*g* for 5 min. Supernatant was discarded from each tube and experiment was resumed with the bacterial pellets. The pellets were re-suspended in 3 ml sodium citrate buffer and centrifuged again. The pellets were again re-suspended in 3 ml buffer containing 1.2 ml (35 mM) ENU solution (prepared in sodium citrate buffer with pH 4.1). The cultures were incubated, separately, for 0, 5, 10, 15, 20, 25 and 30 min. Centrifugation was done at 10,000×*g* for 5 min. Control medium, containing growth culture without mutagen, was also run in parallel with the conditions similar to those of test culture. ENU of each tube was, then, washed with 3 ml of sodium citrate buffer and the resulting pellets were re-suspended in 3 ml of seed culture medium. A 200 µl of each sample was transferred to Petri plates containing the seed culture enriched with methionine and threonine (composition stated above). The cultures were incubated at 30°C for 24 h.

Toxicity during exposure and the number of cell divisions occurring during the expression period were determined by sampling aliquots of cells before and after the exposure and expression time on agar plates. Untreated (control) cultures were carried through similar protocols to determine the spontaneous mutation frequency and to obtain the mutants for subsequent analyses. After repeated trials, lysine over-producing auxotrophs of *B. flavum* were obtained. The colonies, grown on the agar plates, were counted and compared to find out least lethal and most effective concentration of mutagen. Only the auxotrophs survived on the agar plates. Surviving colonies were transferred aseptically to the seed culture medium containing methionine and threonine (2 g/l, respectively). Auxotrophs were grown under optimized fermentation conditions. Then, the mediums were fermented with mutant and wild type *B. flavum* cells for 24 h in the separate flasks. Spectrophotometric determination was done separately, to check the lysine content change in each culture medium.

#### Data analysis

All the data were analyzed by SAS (1998) software. Means of all the parameters and lysine production by the bacterial strain were compared and analyzed through student's *t*-test. The relationship between the parameters and lysine production during each analysis was driven using non-linear regression analysis and P>0.05 was considered to be significant.

## RESULTS AND DISCUSSION

*B. flavum* was selected for hyper-expression of lysine in the present study because it is a good producer of lysine by chemical mutagenesis. Before heading to mutation procedure, growth conditions were optimized and maximum lysine production was analyzed from the wild type microorganism. Optimization was done for the following parameters:

### Effect of carbon source

Carbon sources affect the production of lysine to varying extent in different microorganisms (Tada et al., 2000; Pfefferle et al., 2003; Naz et al., 2001; Brautaset et al., 2003). We tried different carbon sources such as glucose, sucrose, acetate and corn steep liquor for the production of lysine from *B. flavum* to a final concentration of 20 g/l (Yasurou et al., 1991; Ali et al., 2009). Figure 2 shows that the bacterial cells grown on 20 g/l of

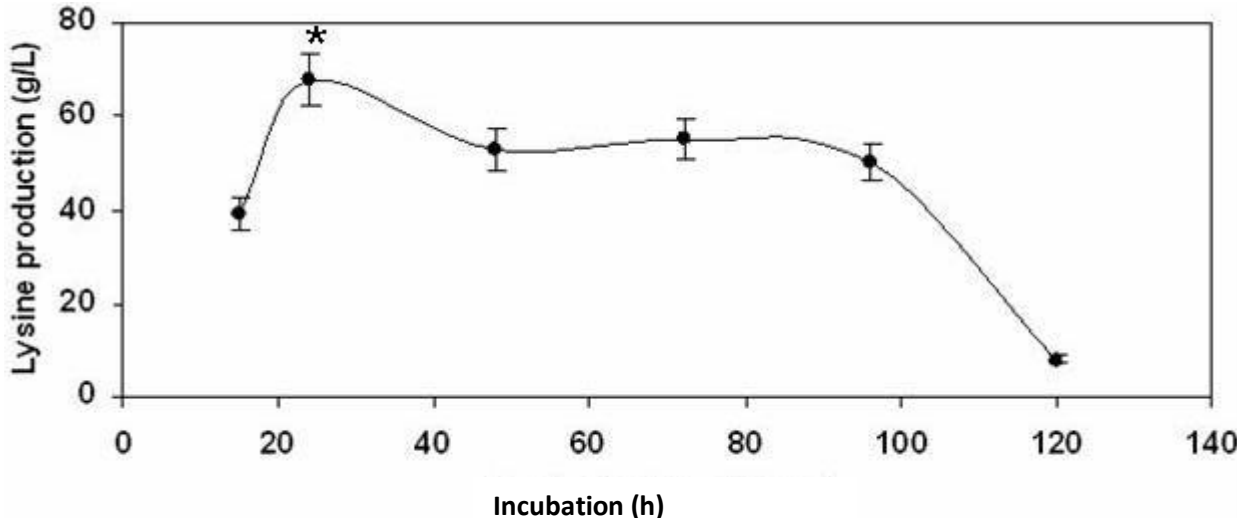


**Figure 3.** Determination of lysine production from *B. flavum* during different time intervals. *B. flavum* was grown for different time intervals (15, 24, 48, 72, 96 and 120 h), to observe optimum fermentation time for lysine production process, by keeping the above studied parameters in optimum state. After getting bacterial culture, the lysine production was seen with UV-Visible spectrophotometry and results were compared with each other to find out the optimum level of fermentation time interval (* shows significant results and P>0.05 was considered to be significant).

corn steep liquor had significantly higher quantities of lysine (50.4 g/l) as compared to sucrose and acetate (3.57 and 14.82 g/l, respectively) because corn steep liquor is a rich source of glucose and other growth enhancing products that can accelerate growth and product yield of microorganisms. Regarding these properties, it is used as the main source of carbon in fermentation biotechnology (Ikeda, 2003; Ali et al., 2009). Figure 2 also demonstrates a comparatively higher lysine production (65.2 g/l) in the presence of 20 g/l glucose as sole substrate as compared to sucrose or acetate (P>0.05). Sucrose and acetate, however, have not been proved as good carbon sources for *B. flavum* culture so far, despite the fact that they were successfully used for the production of valine (Krause et al., 2010) or tryptophan are relevant to all industrial molasses-based production processes using *Corynebacterium glutamicum* (Ikeda, 2003; Krause et al., 2010; Blombach et al., 2010) rather than *B. flavum*. Sucrose uptake in *B. flavum* occurs via a phosphotransferase system (PTS) whereby sucrose is phosphorylated at the glucose ring followed by invertase-catalyzed hydrolysis into glucose-6-phosphate and fructose (Shiio et al., 1990; Woo et al., 2010). Similar mechanisms have also been reported for other microorganisms such as *C. glutamicum, Streptococcus lactis*, *Staphylococcus xylosus* (Wagner et al., 1993) and *Clostridium acetobutylicum* (Tangney and Mitchell, 2000). In contrast to these bacteria, however, *B. flavum* does not possess an ATP-dependent fructokinase for conversion of the liberated intracellular fructose into fructose-6-phosphate. This leads to a rather complex situation for the bacterium to utilize and enter this carbon source into the main metabolism. Fernandez-Gonzale et al. (1996) have reported 26.1 g/l lysine production on glucose source from *Brevibacterium lactofermentum* CB61 (Fernandez-Gonzale et al., 1996). Glucose has been used as the carbon source in almost all metabolic flux studies with *B. flavum* (Kiefer et al., 2004). Only selected investigations have been based on other substrates such as lactate (Cocaign-Bousquet and Lindley, 1995), acetate (Cocaign-Bousquet et al., 1993; Wendisch et al., 2000), sucrose or fructose (Kiefer et al., 2004; Dominguez et al., 1998). Wittmann and Heinzle (2002) have performed quantification of extracellular substrates involving sucrose, glucose, acetate, pyruvate and corn steep liquor for lysine production from *B. flavum* and *C. glutamicum*. Study of this research group revealed that the bacterium showed maximum lysine production (57 g/l) on glucose source as compared to sucrose because the glucose monomer was completely channeled into glycolysis at the level of glucose-6-phosphate.

## Effect of fermentation period

Microorganisms have a specific fermentation period during which they remain viable and produce maximum quantity of metabolites. After this specific period of production and growth, microorganisms start to die and quantity of metabolite production is decreased to a significant amount (Cocaign-Bousquet et al., 1993). During present experiment, the culture was grown for 15, 24, 48, 72, 96 and 120 h. Figure 3 shows that maximum lysine production (67.8 g/l) was obtained in the culture grown for 24 h (P>0.05). Fermentation period for *B. flavum* was, thus, found to be 24 h and after this period, decline phase

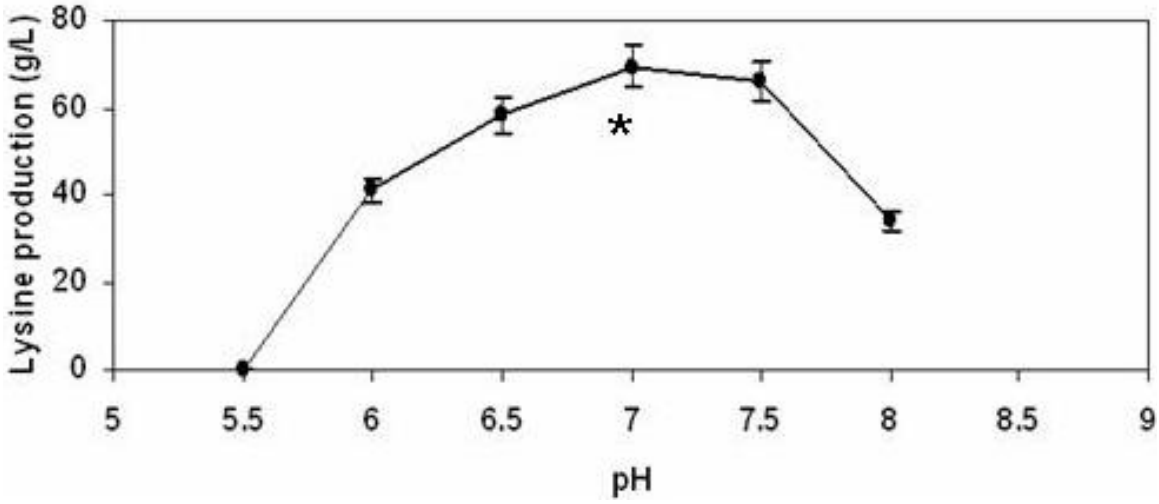


**Figure 4.** Determination of optimum pH for lysine production from *B. flavum.* The effect of different levels of pH (5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) was seen on lysine production from *B. flavum* and after yielding the growth on optimized parameters; the results were constructed with UV-Visible spectrophotometry and compared with each other for seeing optimum level of the parameter under study (* shows significant results and P>0.05 was considered to be significant).

of bacterial life started. Naz et al. (2001) and Ohnishi et al. (2005) cultured *B. flavum* with the same conditions used in present study and found 24 h cultivation time as optimum for *B. flavum*. Takac et al. (1998) have cultured *B. flavum* on the fermentation medium consisting of 25 g glucose. The fermentation was performed at 30°C for 38 h in a 3.5 L bioreactor in batch mode where temperature, pH, foam and aeration rates were controlled but the lysine production (57 g/l) was low as compared to the present study.

## Effect of pH

*B. flavum* was grown at different pH levels: 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. Maximum lysine content was obtained at pH 7.0. Figure 4 shows the effect of different pH levels on lysine production. Bacterial cells grown at pH 7.0 showed 69.5 g/l lysine in growth medium as compared to 58.1, 65.9 and 34.1 produced at pH 6.5, 7.5 and 8.0, respectively (P>0.05).

## Effect of temperature

To evaluate the effect of temperature, the bacterium was grown at 10, 20, 25, 30, 37 and 42°C. Figure 5 shows that 76.15 g/l lysine was observed in the culture grown at 30°C (P>0.05). *B. flavum* is a mesophilic bacterium and all the enzyme activity of this bacterium is optimum at temperatures near 30°C. While, the temperatures above or below 30°C cause the bacterium to lose its enzyme activity and hence the lysine production is decreased. The results obtained during this experiment are in line with the previous workers, who used *B. flavum* for lysine production in batch fermentation analysis (Ohnishi et al., 2005; Tada et al., 2000; Gerova et al., 2011). The culture was performed on a rotary shaker maintained at pH 7.0 and 30°C. Takac et al. (1998) cultured *B. flavum,* for lysine production on the glucose broth at pH 7.3. The fermentation was performed at 30°C for 38 h. Naz et al. (2001) performed the same experiment at 37°C and pH 7.0 and lysine content was 50 g/l.

## Effect of lactate concentration

Lactate is used as an enhancer of lysine production and is added to the growth medium in moderate quantities (Dominguez et al., 1998). Lactate levels, used in the experiment, were 0, 2, 12, 22, 32, 42 and 52 g/l. Figure 6 shows that a significant increase in lysine content was seen in the culture grown with 2 g of lactate (80.2 g/l lysine) as compared to 12, 32, 42 and 52 g/l lactate (P>0.05). Lysine content obtained at the concentrations above 2 g of lactate is as followed: 62.8, 37.6, 36, 7.32 and 3.13 g/l at 12, 22, 32, 42 and 52 g/l lactate concentration, respectively. Above mentioned results reveal that the increased concentration of lactate caused repression of glucose utilization and thus, the decreased lysine content was seen in the cultures grown on 32, 42 and 52 g/l lactate. Decrease in lysine production at higher lactate concentrations might be due to feed back inhibition of higher levels of lactate that reverses the glycolytic pathway and inhibits the utilization of glucose (Ikeda, 2003). Lack of glucose utilization causes whole pathway

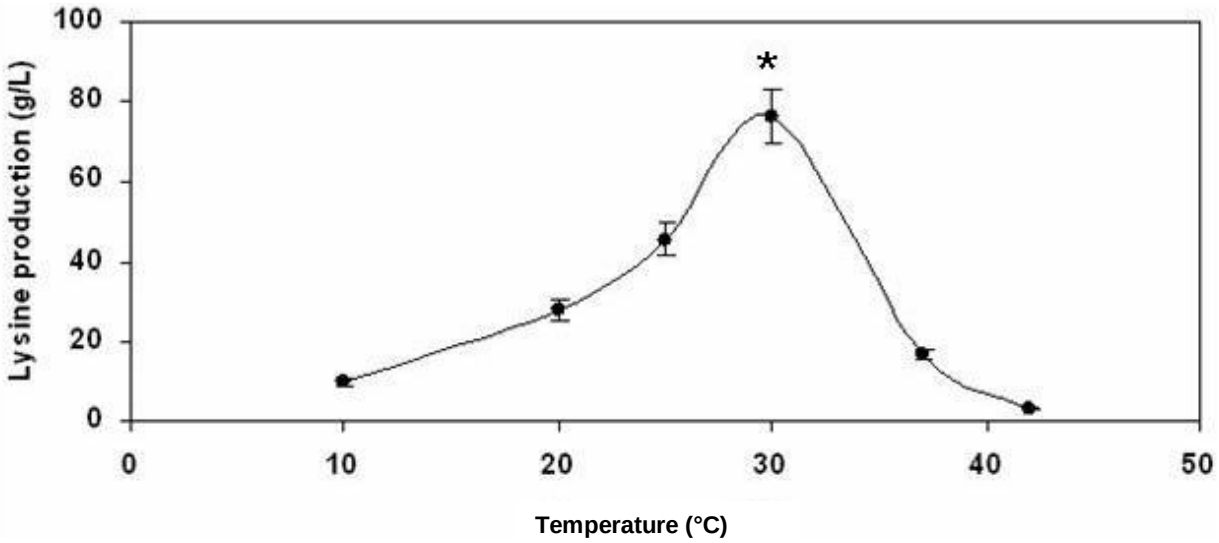


**Figure 5.** Determination of optimum temperature for lysine production from *B. flavum.*
The effect of different temperatures (10, 20, 25, 30, 37 and 43°C) was seen on lysine production from present bacterium and after yielding growth at yet optimized conditions; the results were constructed with UV-Visible spectrophotometry and compared with each other for sorting out optimum level of the parameter under experiment (* shows significant results and P>0.05 was considered to be significant).

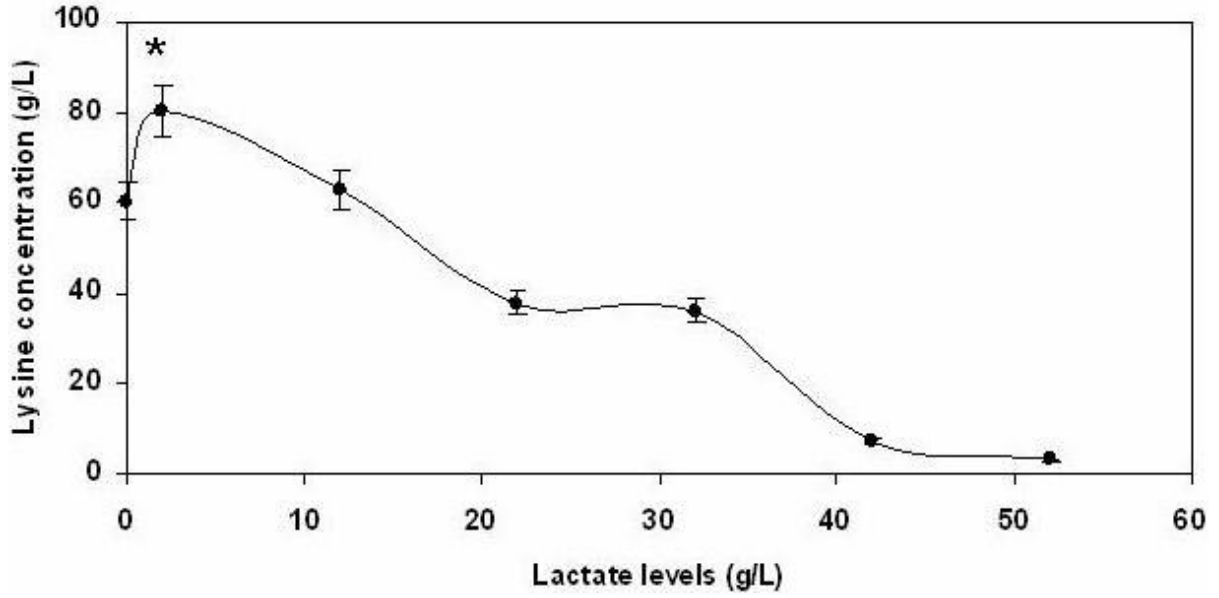


**Figure 6.** Effect of different levels of lactate on lysine production from *B. flavum.* Effect of different levels of lactate was seen on lysine production from *B. flavum* in the following experiment. The bacterium was grown with 0, 2, 12, 22, 32, 42 and 52 g/l of lactate in separate Erlenmeyer flasks and after getting optimum cell growth, results of each lactate concentration were seen and compared with the control experiment (having lactate concentration = Zero), to determine the optimum lactate level that can give maximum lysine production (* shows significant results and P>0.05 was considered to be significant).

to stop that leads to unavailability of substrate to the bacterium. Findings of this experiment showed that low quantity of lactate can enhance substrate utilization capacity of the *B. flavum*, which leads to enhanced lysine production but higher levels have negative effect on the same metabolism. After optimization of all the conditions, 80.2 g/l of lysine was obtained from the wild type bacterium. The lysine concentration obtained during the

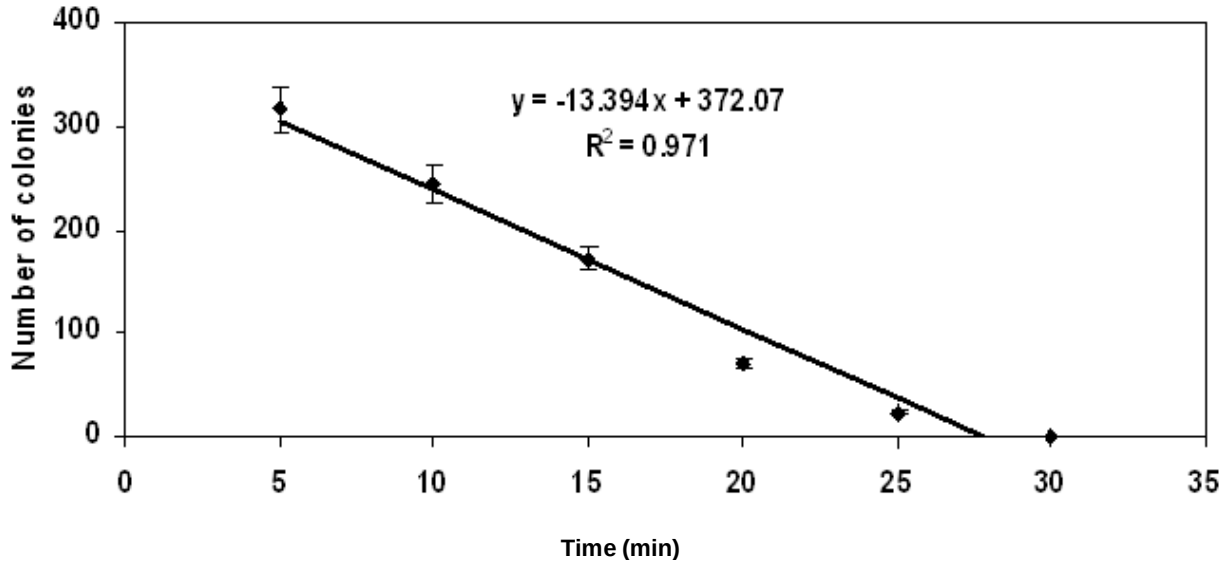


**Figure 7.** Colony count after different times of exposure to N-nitroso-N-ethylurea (ENU). *B. flavum* was grown in the presence of 35 mM ENU for different time intervals (5-30 min ) to find out the least lethal and most effective concentration of ENU for the desired mutation. After 5, 10, 15, 20, 25 and 30 min , living cells (methionine + threonine auxotrophs/ mutants) bacterial cells were grown in seed culture medium (composition mentioned in materials and methods section), supplied with 2 g/l of L-isomers of methionine and threonine, for 24 h and colonies were count in every plate. The plate containing maximum colonies contained appropriate concentration of mutagen.

present study is significantly higher than any concentration reported before. Tada et al. (2000) produced 70 g/l lysine from *B. flavum* in the presence of 1.5 g/l lactate as compared to 54 g/l produced in the absence of lactate and determined that lactate can enhance lysine production capacity of *B. flavum* mutants.

## Mutagenesis of *B. flavum*

The mutation approach has become the most extensively used tool for industrial organisms (Brautaset et al., 2003). We used, for the first time, ENU (35 mM) for mutation of *B. flavum* to hyperexpress the production of lysine. Bacterial cultures were treated with ENU for 5, 10, 15, 20, 25 and 30 min and grown on seed culture medium with methionine and threonine (composition stated in the Materials and Methods section). The culture obtained in the seed culture medium was grown on agar plates for 24 h at 30°C. Colonies were counted after 24 h incubation. Figure 7 shows that the agar plate, treated with mutagen for 5 min, had maximum colonies (316) of the bacterium than that obtained on the plates grown with other concentrations of the mutagen. The cultures treated in the presence of ENU for 10, 15, 20 and 25 min had 244, 172, 71 and 23 colonies ($P>0.001$), while the bacterial cells cultured with ENU under same conditions did not show any growth. These results show that 90-99% of the population was killed on this plate. The surviving bacterial cells (auxotrophs) are of interest because various intermediate compounds associated with the pertinent biosynthetic route tend to accumulate behind a metabolic block. These changes can be identified by a screen and translated through production.

## Effect of homoserine on *B. flavum* auxotrophs

Homoserine is an intermediate product of lysine, methionine and threonine metabolism. *B. flavum* mutants, which are threonine plus methionine auxotrophs, have been shown to secrete substantial quantities of lysine on glucose as a sole carbon source and such mutants are also called homoserine dehydrogenase mutants (Hanson et al., 1996). However, no other mutants that secrete considerable quantities of lysine have been found with this approach (Belitsky, 2002). As the bacterial mutants needed threonine and methionine for normal growth, the effect of these two parameters was also observed. So, *B. flavum,* the auxotrophic cells were grown in the media containing methionine+threonine (2 g/l each), 2 g/l threonine, or 2 g/l methionine and without these amino acids (control experiment). Maximum growth and lysine production (125 g/l) was obtained from the mutants, grown in the culture medium containing regulated quantities of threonine and methionine as compared to 81 g/l produced without the two metabolites ($P>0.001$). Lysine production was increased in the presence of both threonine and methionine and decreased in the absence of both of them or any one of them (Figure 8). So, increased lysine production was achieved through mutagenesis. Results of present study revealed that

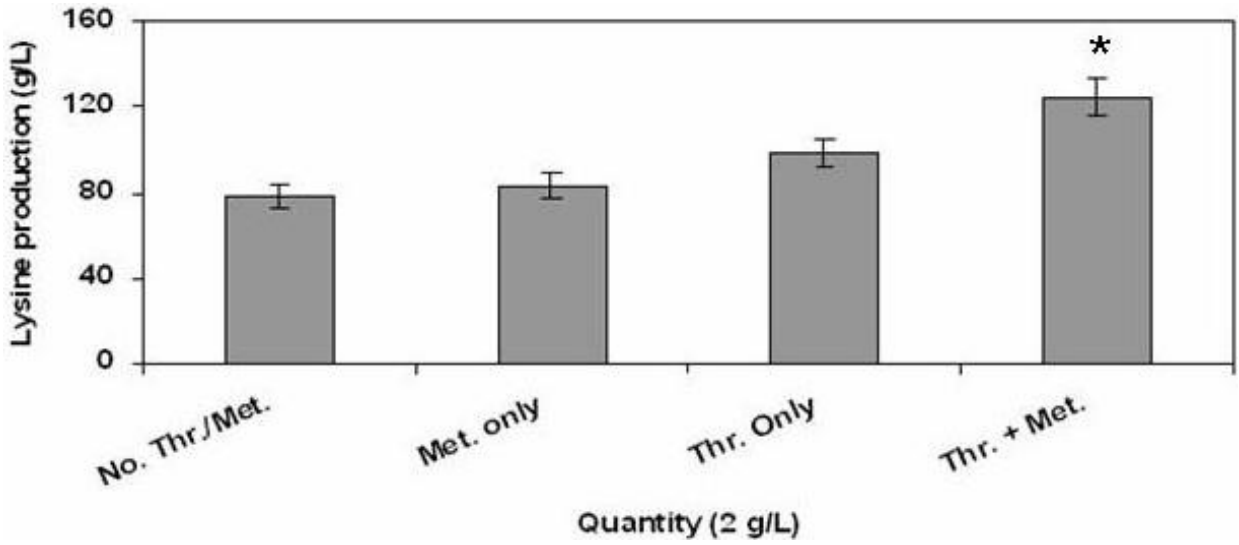


**Figure 8.** Effect of methionine and threonine on lysine production from *B. Flavum* auxotrophs. Bacterial cultures were grown with 2 g/l of L-threonine and L-methionine separately in such sequence: (A) No threonine/Methionine (control), (B) threonine only, (C) methionine only and (D) threonine + methionine, under the conditions optimized before starting mutagenesis. After 24 h of incubation, spectrophotometric assay of lysine was done (at λ= 545 nm) to reveal the effect of two above mentioned amino acids on lysine production from *B. flavum* (* shows significant results and P>0.05 was considered to be significant).

chemical mutagenesis successfully increased lysine production (125 g/l) and the lysine concentration obtained during this study is higher than that reported in previous studies.

Improved lysine production from *B. flavum* was a recurrent theme of presented work and our results have proved that chemical mutagenesis can lead to considerably high quantities of lysine from *B. flavum*. Finally, the most outstanding illustration of the recent study, which led to the improvement of lysine-producing strain, is the coordinated overexpression of several genes (e.g; encoding pyruvate carboxylase and aspartokinase), in realization of the fact that pathway is shared and not localized to any particular enzyme. This resulted in a marked improvement of specific lysine productivity, where many similar efforts had failed in the past. We offer this case study as an example of what can be accomplished by such approaches to strain improvement that can save the use of expensive poultry feeds such as soybean meal. The need for such approaches will be intensified in the near future to generate data from cell-wide and genome-wide measurements.

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