Lysine Production by L-homoserine Resistant Mutant of *Brevibacterium flavum*

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Summary: *Brevibacterium flavum*, an industrially important microorganism used for the production of lysine, was mutated chemically by *iso*-propyl-methane sulphonate to produce an auxotroph with increased yield of lysine. One of the mutant strain *B. flavum* AJ/SA-315 showing maximum lysine activity was selected after screening. Yeast sludge was fermented by this mutant strain of *B. flavum* to increase the lysine content. Maximum lysine concentration of about 57.2 mg/100 mL was achieved with 2 % corn steep liquor. The amino acid profile of final microbial biomass protein showed that it was enriched with all essential amino acids. Fermentation of yeast sludge with mutated *B. flavum* AJ/SA-315 enhanced the lysine content from 1.59 % to 3.71 %. The fermented samples can be a good source of lysine and other essential amino acids for use in synthetic feed.

Introduction

Biotechnological production of amino acids today serves as a market with strong prospects of growth. In the foreground are the fermentation processes, which are now widely established in the production of amino acids [1]. It is expected that the demand for amino acid production will increase in future [2, 3]. Lysine is nutritionally essential for humans and animals. It cannot be synthesized internally but may be added to food and feed materials to improve the protein quality [4, 5]. It is used mostly as a feed additive at a rate of above 6×10^5 tones/year [6]. The lysine market has increased successively to a current annual market volume of about 750,000 tones [7, 8]. Various free-living bacteria that are capable of exerting beneficial effects on plants have potential for use in agriculture [9, 10]. Advances in fermentation technology and strain improvement of amino acid producing micro-organisms [11] have enabled industrial scale production of lysine [6, 12].

L-lysine is produced by many bacteria including *Brevibacterium flavum* [13], *B. lactofermentum* [14], *Bacillus subtilis* and *C. glutamicum* [15, 16]. *B. flavum* is an industrially important microorganism used in the production of lysine.

The increased production of waste in the world is of great concern at different levels of population [17, 18]. These waste products can be used by fermentation for the production of useful products. Feed industry has always been in need of cheap and quality protein sources, but with the expansion and increase in production capacity of this industry, these sources remain short in supply and hence of higher cost. This situation triggered the search for novel protein sources and development of scientific methods to enhance the nutritive value of existing non-conventional feed resources either by mechanical, chemical, or biotechnological methods. Yeast sludge protein is lysine deficient; hence, it is imperative to enrich it with lysine prior to poultry feeding. Enrichment of distillery sludge with lysine can be done by fermenting it with lysine-producing strain of *B. flavum*. The biomass thus produced will be of low cost with higher quality protein which can be fed to poultry and livestock after proper management.

In this paper, we report a novel chemical mutation of a *B. flavum* strain designated as *B. flavum* AJ/SA-315, and its optimization to enhance the lysine content. The strain can be utilized in the hyperproduction of lysine for use in food and feed.

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Results and Discussion

Brevibacterium flavum was mutated by a chemical mutagen *iso*-propyl-methane sulphonate. Colonies treated with *iso*-propyl-methane sulphonate were again selected on L-homoserine. Colonies resistant to L-homoserine were picked up and auxotroph mutants *of B. flavum* were tested for lysine activity. Colony count of L-homoserine resistant strain was done by using counting chambers under Olympia Microscope. Out of 70 colonies tested, one colony designated as *B. flavum* AJ/SA-315 showed maximum lysine concentration of 35 mg/100 mL (detail data not shown). L-homoserine resistant mutant strain *B. flavum* AJ/SA-315 was used to optimize fermentation conditions to further increase lysine concentration and to ferment yeast sludge for enhanced lysine production.

Optimal Conditions for Lysine Production by B. flavum AJ/SA-315

With a view to get enhanced lysine production, different fermentation conditions were optimized. Initially studies were performed on micro shake flasks to optimize fermentation conditions for the production of lysine through fermentation of yeast sludge with *B. flavum* AJ/SA-315. The fermentation temperature was maintained at 37 °C and pH 7 throughout the fermentation period (3 days), unless otherwise mentioned. The orbital shaker was operated at a speed of 150 rpm.

Influence of Optimum Substrate Concentration

Among different substrate (yeast sludge) concentrations, 80 % (w/v) yeast sludge supported higher lysine production (Fig. 1). Lysine production did not increase with further increase in substrate concentration.

Effect of Carbon: Nitrogen Ratio on Lysine Production

Carbon nitrogen ratio in fermentation process influences fermentation of protein concentrates. To explore the influence of this variable on production of lysine, ratios were obtained by increasing the urea concentration in the medium. A ratio of 1:4 supported maximum values of 48.7 mg/100 mL of lysine. The lysine production decreased after that at 1:6 and 1:5 ratio (Fig. 2).

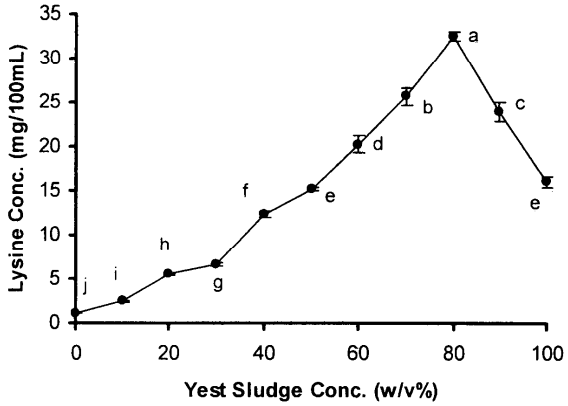

Fig. 1: Effect of various yeast sludge concentrations on lysine production from *B. flavum* AJ/SA-315 at pH 7 and 37 °C. Error bars show standard deviation among three observations. Means sharing similar letters are statistically non-significant (P>0.05).

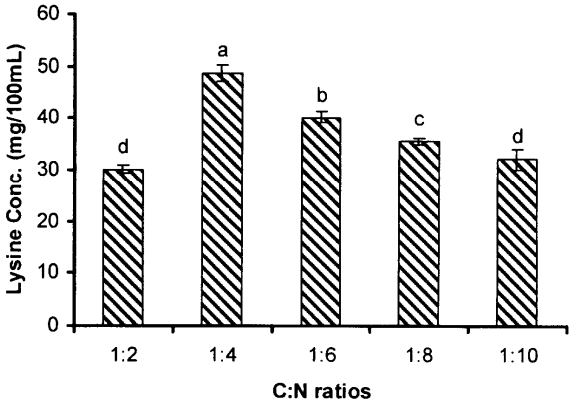

Fig. 2: Effect of different C:N ratio on lysine production by *B. flavum* AJ/SA-315 grown on yeast sludge. Error bars show standard deviation among three observations. Means sharing similar letters are statistically non-significant (P>0.05).

Generally the results confirmed that urea; a low cost fertilizer, supported maximum lysine production and confirmed the findings of Hashmi [19].

Effect of Supplementation with Corn Steep Liquor on Lysine Production

Corn steep liquor, a cheap nitrogen source, was added to the fermentation medium under

previously optimized conditions to enhance lysine production. The results show that 2 % corn steep liquor gave maximum lysine production (Fig. 3). Generally, the results confirmed that corn steep liquor, a low-cost by-product of the starch industry, supported the maximum lysine production from *B. flavum* AJ/SA-315.

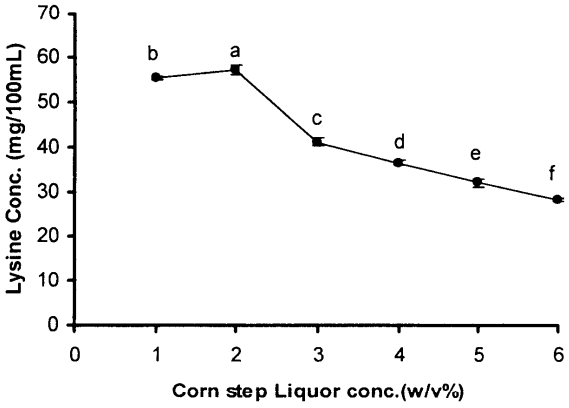

Fig. 3: Influence of corn steep liquor on lysine production by *B. flavum* AJ/SA-315. Culture under optimal conditions pH 7; temperature 37 °C; incubation time 72 h; 80 % yeast sludge, C:N ratio 1:4. Error bars show standard deviation among three observations. Means sharing similar letters are statistically non-significant (P>0.05).

In recent decades optimization of fermentation strategies have led to increase yield and rates of industrial amino acid production. Since costs of substrates play an important role in industrial amino acid fermentations therefore, manufacturers aim to use the cheap raw materials [20]. For industrial fermentations use of low cost and complex sugar substrates such as, cereal brans, agriculture fruit wastes, crop residues and molasses are common, however their usage depends on their geographical locations [21, 22]. Lysine production is strongly affected by growth rate of strain and culture conditions [23]. Therefore, different fermentation conditions of auxotroph mutant of *B. flavum* AJ/SA-315 were optimized to increase lysine concentration in this study.

Hence, it is concluded from this study that corn steep liquor 2 % at C:N ratio of 1:4 at pH 7, 37 °C and 78 h of incubation with 80 % yeast sludge as a substrate were found to be optimal for lysine production from *B. flavum* AJ/SA-315. These optimized conditions were then employed to ferment yeast sludge on a higher scale with *B. flavum* AJ/SA-315.

Chemical Analysis of the Yeast Sludge

Chemical analysis of the yeast sludge before and after fermentation with *B. flavum* under the optimized conditions are presented in Table-1. Fermentation of yeast sludge with mutated *B. flavum* AJ/SA-315 not only increased the lysine concentration but also increased the values of crude and true proteins (Table-1). Crude protein in the biomass was increased after fermentation process. It was 29.75 % in the yeast sludge and increased to 30.31 % in the biomass produced.

Table-1: Chemical analysis of the yeast sludge on dry weight basis before and after fermentation with *B. flavum* AJ/SA-315

Components	Before fermentation (Yeast sludge)	After fermentation (Biomass)
Moisture	73.8 % ± 1.93 [a]	11.3 % ± 0.38 [b]
Crude protein	29.75 % ± 0.74 [b]	30.31 % ± 0.64 [a]
Crude fiber	0.0 % ± 0.0	0.0 % ± 0.0
Ash	30.0 % ± 0.17 [a]	28.6 % ± 0.11 [b]
Crude fat	1.2 % ± 0.03 [b]	1.4 % ± 0.02 [a]
Mannan-oligosacchrides	4.6 % ± 0.09 [a]	4.3 % ± 0.07 [b]
Nitrogen free extract	34.45 % ± 0.61 [a]	35.09 % ± 0.43 [a]
RNA	1.82 % ± 1.12 [a]	1.75 % ± 0.03 [a]

Each value is a mean of three replicates ± stands for standard deviation among three independent analyses. Paired T-test was applied to compare the results before and after fermentation. Means sharing similar letters in a row are statistically non-significant (P>0.05).

The value of true protein in yeast sludge was 20.78 % and was increased to 22.12 % in the biomass produced after yeast sludge was fermented with *B. flavum* AJ/SA-315.

Amino Acid Profile

Amino acid composition of yeast sludge was determined on amino acid analyzer before and after fermentation with *B. flavum* AJ/SA-315 (Table-2). Lysine content was limited in yeast sludge before fermentation. Fermentation of yeast sludge with *B. flavum* significantly increased the lysine content. It was 1.59 % in the yeast sludge and increased up to 3.71 % in the biomass produced after fermentation of yeast sludge with *B. flavum*. Hence, lysine was not limiting amino acid after the fermentation. The biomass thus produced contains sufficient lysine and meet the requirement of poultry and livestock.

Table-2: Amino acid profile of yeast sludge before and after fermentation with mutated *B. flavum AJ/SA-315* (only essential amino acid profile shown).

Amino acid	Amino acid (%) in Yeast sludge (without fermentation)	Amino acid (%) in biomass protein (after fermentation)
Threonine	3.02	5.7
Valine	3.61	3.46
Leucine	4.32	4.12
Isoleucine	3.72	3.46
Methionine	1.98	1.96
Phenylalanine	3.35	3.24
Lysine	1.59	3.71
Arginine	5.39	4.27
Histidine	1.89	1.90

Test samples were hydrolyzed with HCl and analyzed on an automated amino acid analyzer

Earlier, many scientists have used different species of *Brevibacterium* for lysine production. Nine different species of the genus *Brevibacterium* were tested for their ability to use starch as a substrate for growth for lysine production. However, none of the nine strains of *Brevibacterium* were able to use starch as carbon source for lysine production [20]. Tsuchida *et al.* [24] reported *Brevibacterium lactofermentum* ATCC13869 as a parent of lysine producing strain. Similarly high lysine producing strains were obtained by derivation of fluropyruvate (FP)-sensitive mutants from *B. lactofermentum* AJ3990 [25]. Mutagenesis of *B.* has also been reported. *B. flavum* is also well known for the production of lysine. Lysine was produced 41 g/L by a mutant strain No 1-231 of *B. flavum* [26]. Similarly, 36 g/L lysine was produced by *B. flavum* AJ12429 [11]. Likewise, Shiio *et al.* [27] found that 50 g/L lysine was produced by a mutant strain No. 22 of *B. flavum* after 72 h incubation in medium containing soyabean meal hydrolysate, methionine and 100 g/L of glucose. Lysine production and biosynthesis was studied from *B. flavum* 115 [28]. Lysine biosynthesis increased in *B. flavum* 115 cells under stringent response, induced by threonine limitation.

This study supports the fact that lysine production can be improved by using agricultural by products as carbon sources during fermentation. The cheap prices and availability of raw materials (yeast sludge and corn steep liquor) have great potential for the industrial production of lysine. Hence in this study it was concluded that yeast sludge and corn steep liquor can be used to enhance lysine production from *B. flavum* AJ/SA-315 without costly pretreatment or nutrient supplementation. The final yeast biomass is a protein-rich animal feed material. The demonstration of its successful production at laboratory level is an incentive to develop an effective large scale method for the production of microbial protein using cane molasses and corn steep liquor. The present results contribute an increase in the relevant information concerning lysine production from waste products.

Experimental

Microorganism and Mutagenesis

B. flavum was taken from stock cultures of Department of Animal Nutrition, University of Agriculture, Faisalabad, Pakistan. This strain was mutated to increase the lysine concentration. Mutated strain was maintained on nutrient agar medium (2 g/L yeast extract, 5 g/L peptone, 5 g/L sodium chloride, 10 g/L glucose and 25 g/L agar) pH was adjusted to 7. Chemical mutagenesis was applied on the *B. flavum* with mutagen iso-propyl-methane sulphonate (molecular weight 138.19) from Eastern Organic Chemicals, USA. Mutated colonies of *B. flavum* were transferred aseptically on the medium containing L-homoserine as auxotrophic requirement. Only the auxotrophs of the *B. flavum* could survive on the medium. Auxotroph resistant mutants to homoserine were grown and lysine concentration was determined. One of the L-homoserine resistant mutant strain showing maximum lysine concentration designated as *B. flavum* AJ/SA-315 was grown and its fermentation conditions were optimized with respect to lysine.

Media and Culture Conditions

For inoculum preparation of mutated *B. flavum* AJ/SA- 315 glucose broth medium was used (yeast extract, 2 g; peptone, 5 g; NaCl, 5g; glucose, 10 g in 1 liter distilled water at pH 7) [29]. Inoculum was grown on an orbital shaker at 37 °C (120 rpm for 48 h). Concentration of the organism was adjusted to an optical density (OD) of 0.6 at 610 nm by diluting the suspension with sterile distilled water to get the homogenous suspension containing 4×10^7 cells/mL [30]. This suspension was used as inoculum for the fermentation medium. The biomass was produced in the fermentation medium under the same conditions as discussed above. The pH of the fermentation medium was adjusted to 7 and temperature 37 °C after 72 h with shaking at 150 rpm to optimize different fermentation conditions for lysine production. 80 % yeast sludge was used as a substrate.

Biomass was harvested by centrifugation at 10,000 rpm for 20 min at 4 °C [31]. Resulting supernatant was tested for lysine activity according to the method of Chaves *et al.* [32].

Influence of Carbon: Nitrogen Ratio on Lysine Production

Based on the results obtained in the above experiment, another experiment was conducted in which different ratios of carbon to nitrogen (1:2, 1:4, 1:6, 1:8 and 1:10) were tested to get the maximum biomass lysine. The carbon to nitrogen ratio at which maximum lysine production occurred was considered as optimum for the growth of *B. flavum* AJ/SA- 315. It was maintained in the subsequent experiments.

Influence of Corn Steep Liquor on Lysine Production

Corn steep liquor (CSL) concentration was optimized to different concentrations of corn steep liquor (1, 2, 3, 4, 5 and 6 w/v %) and were tested to get the enhanced lysine production from mutated *B. flavum* AJ/SA-315.

Analysis of the Yeast Sludge

Yeast sludge was obtained from Shakargang Mills Ltd., Jhang, Pakistan. Analysis of the yeast sludge was performed before and after fermentation with mutated *B. flavum* AJ/SA-315, following A.O.A. C Method [33] to find out the nutritive value of the yeast sludge.

Amino Acid Profile

Amino acid composition of yeast sludge was determined on amino acid analyzer before and after fermentation with *B. flavum* AJ/SA-315 according to Moore and Stein [34].

Statistical Analysis

The data obtained was subjected to Analysis of Variance Techniques [35] and, in case of significant differences, a Duncan's Multiple Range Test (DMR) was applied [36]. Paired T-test was conducted as described in Steel and Torrie [35].

Conclusion

The fermentation conditions for the microbial production of lysine by *B. flavum* AJ/SA-315 were successfully optimized on laboratory scale. Yeast sludge (80 % w/v) has shown excellent potential as a substrate for lysine production. 2 % corn steep liquor gave maximum lysine production with 80 % substrate (yeast sludge), C:N ratio of 1:4, pH 7, at 37 °C for 72 h incubation in orbital shaker. The biomass protein contains fairly good quality protein rich in all essential amino acids.

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