Fermentation of Yeast Sludge with *Brevibacterium flavum* to Enhance Lysine Concentration

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Summary: *Brevibacterium flavum* is an industrially important microorganism that is used for the production of lysine. Fermentation conditions of *B. flavum* were optimized for enhanced production of lysine. Yeast sludge was fermented by *B. flavum* under the optimized conditions to increase the lysine content. The optimum pH, temperature and incubation time for *B. flavum* for lysine production were 7, 35 °C and 48 h respectively. Maximum lysine concentration was achieved with 1 % molasses, 4 % corn steep liquor and 25 mg % protein hydrolysate of sesame meal. Fermentation of yeast sludge with *B. flavum* increased the lysine content from 1.54 % to 4.78 % as shown by amino acid analysis.

Introduction

Biotechnological production of amino acids, today, has market with great prospects of growth. In the foreground are the fermentation processes, which are now widely established for the production of amino acids [1]. It is expected that the demand for amino acid production will increase in the future [2, 3]. Lysine is nutritionally essential for humans and animals. It cannot be synthesized inside the body 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 tonnes/year [6]. It has to be available in sufficient amounts in feeds and food to meet the nutritional requirements of animals and humans. This supplementation is realized by the direct addition of lysine and, as a result, a tremendous growth in the market has taken place in the past ten years. Lysine is supplemented in poultry especially when cereal based feed is formulated to improve protein quality [7]. In many parts of the world, the requirement for lysine is met through importation [8]. The lysine market has increased successively to a current annual market volume of about 750,000 tonnes [9-11]. Owing to the exploitation of new uses of lysine in pharmaceuticals, cosmetics and polymer materials, the market shows a growth potential of 7–10 % per year [2].

Advances in fermentation technology and strain improvement of amino acid producing microorganisms have enabled industrial scale production of lysine [6, 12, 13]. Fermentative methods seem to be most economical and practicable means of producing lysine and many of such processes have been investigated [14-16]. Lysine was discovered to be produced by many bacteria including *B. flavum* [17], *B. lactofermentum* [18], *B. subtilis* and *Corynebacterium glutamicum* [19]. The bulk of lysine production through out the world depends on direct fermentation of carbohydrates by *Corynebacteria* and *Brevibacteria* species in batch cultures [20]. *B. flavum*, *B. lactoferemntum* and *C. glutamicum* have been used for the last fifty years for the industrial production of different amino acids [21]. *Brevibacterium flavum* is an industrially important microorganism used for the production of lysine.

For fermentation processes, intensive raw material use (such as amino acids, organic acids, and ethanol), process yield (product produced/substrate consumed), in addition to productivity are critical measures of performance and economic viability [22]. Yeast sludge a byproduct of brewing industry, has attracted the attention of scientists. 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 costly. This

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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 yeast sludge with lysine can be done by fermenting it with lysine-producing strain of *B. flavum*. The biomass produced thus will be of low cost and higher quality protein which can be fed to poultry and livestock. In this paper we report increase in lysine concentration through fermentation of yeast sludge with *B. flavum*.

Results and Discussion

Optimum Conditions for Lysine Production from B. flavum

Studies were performed in shake flasks to produce lysine to optimize the fermentation conditions.

Effect of Substrate Concentration on Lysine Production

Among different substrate concentrations (yeast sludge), 40 % substrate concentration supported significantly higher lysine production (Fig. 1) and was found adequate to support higher crude protein. Gradual reduction in lysine concentration was observed when substrate concentration was increased or decreased.

Analysis of variance table (Table-1) showed that there is highly significant difference among substrate levels regarding lysine values. After applying Duncan's Multiple New Range (DMR) test, it became clear that at 40 % concentration of substrate, the lysine value was maximum and statistically significant different from all other levels of substrate. It is also clear from DMR test that lysine value is significantly increased at each level of substrate till 50 w/v % level and after that significantly decreases till 70 w/v % level of substrate. After that *i.e.*, from 70 to 100 w/v % substrate level, the lysine value remains statistically unchanged (Fig. 1).

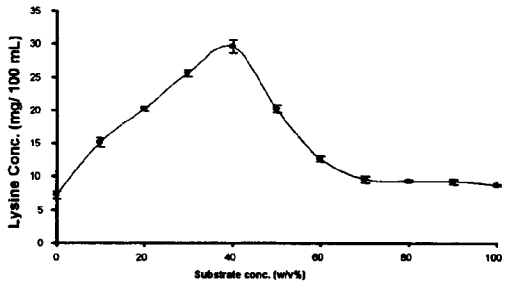

Fig. 1: Effect of various substrates (yeast sludge) to water ratio on lysine production by *B. flavum* at pH 7 and 35 °C. Error bars show standard deviation among three observations.

Effect of Incubation Time, pH and Temperature on Lysine Production

Optimum time course for lysine production was investigated in shake flasks in growth medium with 40 % yeast sludge as a substrate. Fermentation was carried out at 35 °C at pH 7. As shown in Fig. 2

Table-1: Analysis of variance (mean squares).

Source	Substrate (Yeast Sludge) Conc.		Time		pH		Temperature		Molasses		Corn Steep Liquor		Protein Hydrolysate	
	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.
Between	10	174.15**	6	118.10**	5	42.27**	6	194.92**	7	383.47**	5	81.05**	6	404.0**
Within	22	0.293	14	0.725	12	1.35	14	1.75	16	6.39	12	6.84	14	12.2
	Level	Mean	Level	Mean	Level	Mean	Level	Mean	Level	Mean	Level	Mean	Level	Mean
	0	7.2 G	12	12.21 F	5.5	25.10 D	30	32.31 C	0.5	75.3 B	1	64.4 CD	5	58.0 E
	10	15.1 D	24	18.52 E	6.0	29.30 C	35	42.71 A	1.0	82.5 A	2	67.4 BC	10	65.0 D
	20	20.2 C	36	26.22 B	6.5	32.10 B	40	38.61 B	1.5	75.0 B	3	70.4 B	15	75.0 C
	30	25.6 B	48	31.58 A	7.0	34.51 A	45	34.21 C	2.0	69.0 C	4	76.5 A	20	85.0 B
	40	29.5 A	60	24.24 C	7.5	26.29 D	50	24.31 D	2.5	60.0 D	5	65.0 CD	25	92.0 A
	50	20.2 C	72	21.24 D	8.0	26.20 D	55	23.20 D	3.0	58.0 DE	6	62.0 D	30	81.0 BC
	60	12.6 E	84	18.33 E			60	22.21 D	3.5	55.0 EF			35	76.0 B
	70	9.5 F							4.0	51.0 F				
	80	9.3 F												
	90	9.2 F												
	100	8.6 F												

** = Highly significant (P<0.01)

Means sharing similar letters in a cell are statistically non-significant (P>0.05).

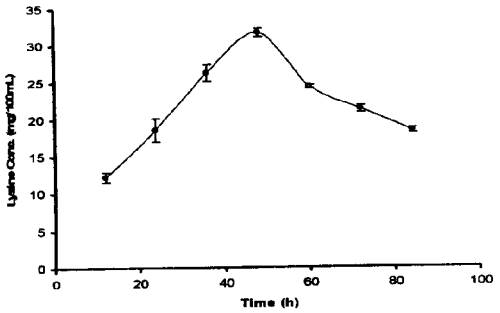

Fig. 2: Time course of lysine production by *B. flavum* at pH 7 and 35 °C. Error bars show standard deviation among three observations.

maximum lysine concentration (31.58 mg/100 mL) was obtained at 48 h. Further incubation resulted in decrease in lysine concentration. Table-1 showed that there is highly significant difference among different time periods regarding lysine production. After applying DMR test, it became clear that at 48 h, lysine value was maximum and significantly different from all other time periods.

Lysine was produced from *B. flavum* at pH range of 6-7 with maximum lysine concentration of 34 mg/ 100 mL at pH 7. Table-1 showed that there is highly significant difference among different pH values regarding lysine production. After applying DMR test, it became clear that at pH 7, lysine concentration was maximum and significantly different from all other values of pH (Fig. 3).

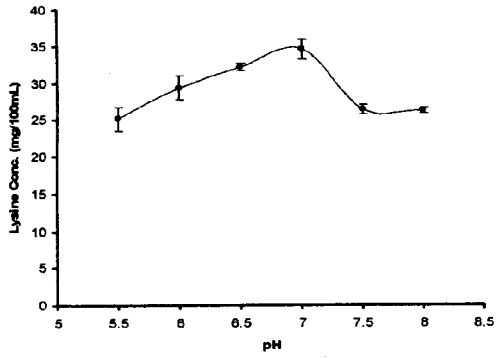

Fig. 3: Effect of pH on lysine production by *B. flavum*. Error bars show standard deviation among three observations.

The temperature of the fermentation medium was one of the critical factors and had profound influence on the production of end product. The production of lysine by *B. flavum* in fermentation medium at different temperatures (30-65 °C) was carried out. Maximum lysine concentration (42.75 mg/ 100 mL) was realized when the fermentation temperature was maintained at 35 °C. Further increase in temperature resulted in decrease in lysine concentration (Fig. 4). There is highly significant difference among different temperature levels regarding lysine values (Table-1). After applying DMR test, it became clear that at 35 °C, lysine concentration was maximum and significantly different from all other levels of temperature.

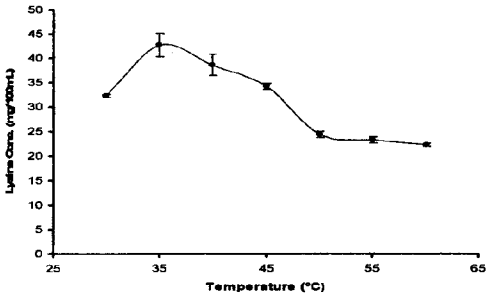

Fig. 4: Effect of temperature on lysine production by *B. flavum*. Error bars show standard deviation among three observations.

Hence optimum pH 7 and optimum temperature 35 °C were used in all the subsequent experiments.

Effect of Molasses, Corn Steep Liquor and Protein Hydrolysate on Lysine Production

Cultivation of *B. flavum* on growth medium containing molasses ranging from 0.5 to 4 % (w/v) showed that maximum lysine concentration of about 82.5 mg/100 mL was achieved with 1 % molasses. Addition of higher concentrations of molasses resulted in low lysine production, it reached the baseline with 4 % molasses (Fig. 5). Analysis of variance table (Table-1) showed that there are highly significant differences among different molasses concentrations regarding lysine production. After applying DMR test, it became clear that at 1 % molasses, lysine concentration was maximum and significantly different from all other concentrations of molasses.

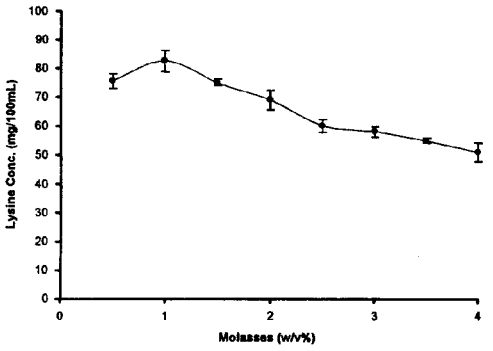

Fig. 5: Influence of molasses on lysine production by *B. flavum*. pH 7; temperature 35 °C; incubation time 48 h; 40 % substrate (yeast sludge) concentration. Error bars show standard deviation among three observations.

Corn steep liquor 4 % and 25 mg % protein hydrolysate of sesame meal were found to be optimum for maximum lysine production from *B. flavum* at pH 7, 35 °C and 48 h incubation. When corn steep liquor was used to enhance the lysine concentration, its concentration reached a maximum of 76.5 mg/100 mL with 4 % corn steep liquor, however no increase in lysine concentration took place beyond this (Fig. 6). Analysis of variance table (Table-1) showed that there is highly significant difference among corn steep liquor levels regarding lysine values. After applying DMR test, it became clear that at 4 % corn steep liquor, lysine concentration was maximum and significantly different from all other concentrations of corn steep liquor. The results confirmed that corn steep liquor, a low cost by-product of the starch industry, supported the maximum lysine production. When different concentrations of protein hydrolysate of sesame meal (5-35 % w/v) were tested to enhance the lysine production, maximum lysine concentration of 92 mg/mL was achieved with 25 mg % protein hydrolysate. Further increase in the protein hydrolysate concentration was not fruitful in lysine production (Fig. 7). Analysis of variance table (Table-1) showed that there is highly significant difference among protein hydrolysate levels regarding lysine values. After applying DMR test, it became clear that at 25 % protein hydrolysate, lysine concentration was maximum and significantly different from all other concentrations of protein hydrolysate.

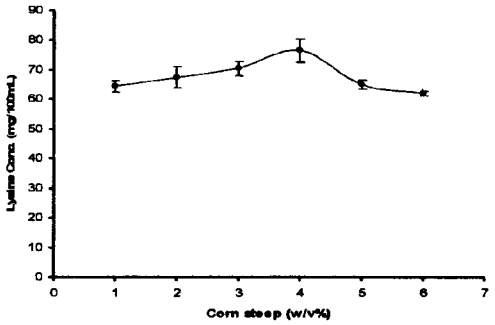

Fig. 6: Influence of corn steep liquor on lysine production by *B. flavum*. Culture under optimal conditions pH 7; temperature 35 °C; incubation time 48 h; 40 % substrate (yeast sludge) concentration; 1% molasses. Error bars show standard deviation among three observations.

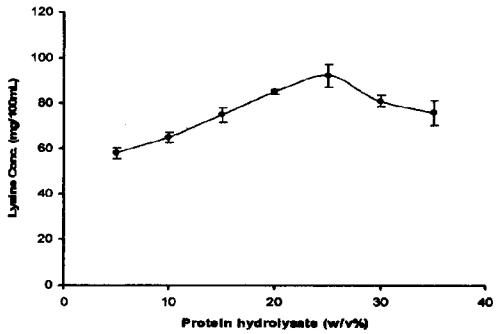

Fig. 7: Influence of protein hydrolysate of sesame meal on lysine production by *B. flavum*. Culture under optimal conditions pH 7; temperature 35 °C; incubation time 48 h; 40 % substrate (yeast sludge) concentration; 1 % molasses; 4 % corn steep liquor. Error bars show standard deviation among three observations.

In recent decades optimization of fermentation strategies led to increased yield and rates of industrial amino acid production. Since costs of substrates played an important role in industrial amino acid fermentations, manufacturers aim to use the cheap raw materials [23]. For industrial fermentations use of low cost and complex sugar substrates such as, cereal brans, fruit wastes, crop residues and molasses are common, however their usage depends on their geographical location [12, 24-27]. Molasses had been reported widely for the

production of lysine [11]. Lysine production is strongly affected by growth rate of strain and culture conditions [28]. Therefore, different fermentation conditions of *B. flavum* were optimized to increase lysine concentration in this study.

Hence, in this study it is concluded that 40% substrate (yeast sludge) concentration 1% molasses, corn steep liquor 4% and 25 mg % protein hydrolysate of sesame meal at pH 7, 35 °C and 48 h of incubation were found to be optimal for lysine production from *B. flavum*. These optimized conditions were then employed to ferment yeast sludge on a larger scale with *B. flavum*.

Proximate Analysis of the Yeast Sludge

Proximate analysis of the yeast sludge and its fermented product were carried out on dry weight basis following A.O.C Methods [29] to find out the relative improvement in the fermented product as shown in Table-1.

Fermentation of yeast sludge with *B. flavum* not only increased the lysine concentration but also increased the values of crude protein (Table-2). Crude protein in the biomass was increased after fermentation process. It was 26.25 % in the yeast sludge and increased to 29.23 % in the biomass produced. The value of true protein in yeast sludge was 21.2 % ± 0.27 and increased to 24.95 % ± 0.36 in the biomass produced by fermentation with *B. flavum*.

Table-2: Proximate analysis of the yeast sludge on dry weight basis before and after fermentation with *B. flavum*

Components	Before Fermentation (Yeast Sludge)	After Fermentation (Biomass)
Moisture	72.3 % ± 1.82	10.3 % ± 0.38
Crude protein	26.25 % ± 0.74	29.23% ± 0.64
Crude fat	1.1 % ± 0.02	1.2 % ± 0.02
Crude fiber	0.0 % ± 0.00	0.0 % ± 0.00
Ash	31.6 % ± 0.14	36.5 % ± 0.11
Nitrogen Free extract	41.05 % ± 0.21	33.07% ± 0.32

Each value is a mean of three replicates ± stands for standard deviation among three independent analyses.

Amino Acid Profile

Amino acid composition of the yeast sludge and its fermented product were determined on amino acid analyzer (Table-3). Lysine content was limited in yeast sludge before fermentation. Lysine concentration was increased in the biomass produced. It was 1.54 % in the yeast sludge and 4.78 % in the biomass produced after fermentation of yeast sludge with *B. flavum*. The biomass thus produced contains sufficient lysine to meet the requirement of poultry and livestock.

Table-3: Amino acid profile of yeast sludge before fermentation and after fermentation with *B. flavum*.

Amino acid	Amino acid (%) in Yeast Sludge (Before Fermentation)	Amino acid (%) in Biomass Protein (After Fermentation)
Valine	0.45	0.50
Leucine	0.94	0.96
Isoleucine	2.31	2.44
Lysine	1.54	4.78
Threonine	1.69	1.65
Methionine	0.04	0.02
Phenylalanine	0.08	0.07
Histidine	1.82	1.83
Arginine	0.95	0.78

Test samples were hydrolysed with HCl and analyzed using an amino acid analyzer.

Many scientists have used different species of *Brevibacterium* for lysine production earlier. 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 [23]. Tsuchida *et al.* [30] reported *B. 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. lacto-fermentum* AJ3990 [31].

B. flavum is well known for the production of lysine. Lysine (41 g/L) was produced by a mutant strain No 1-231 of *B. flavum* [32]. Similarly, 36 g/L lysine was produced by *B. flavum* AJ12429 [12]. Likewise Shi *et al.* [33] found that lysine (50 g/L) 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. Similarly lysine production and biosynthesis was studied from *B. flavum* 115 [34]. 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, molasses, and corn steep liquor) have great potential for the industrial production of lysine. From

the above results we conclude that optimization results in significant increase in lysine production from *B. flavum* and the lysine enriched biomass produced by fermentation of yeast sludge with *B. flavum* contained sufficient lysine that can be used in poultry and livestock.

Experimental

Chemicals

All the chemicals used were of analytical grade unless otherwise stated. Yeast sludge was obtained from Shakargang Mills Ltd., Jhang, Pakistan.

Microorganisms

The strain utilized in the present study was *B. flavum* which was taken from stock cultures of Department of Animal Nutrition, University of Agriculture, Faisalabad, Pakistan. The strain was maintained on nutrient agar medium (NICMB, 1990) consisting of 2 g/L yeast extract, 5 g/L peptone, 5 g/L sodium chloride, 10 g/L glucose and 25 g/L agar.

Media and Culture Conditions

For inoculum preparations of *B. flavum* 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) [16]. Inoculum was grown on an orbital shaker at 35 °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 [35]. 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. Biomass was harvested by centrifugation at 10,000 rpm, for 20 min at 4 °C [36]. Resulting supernatant was tested for lysine activity according to the method of Chaves *et al.* [37].

Optimum Substrate Water Ratio, Incubation Time, pH and Temperature for Lysine Production from Brevibacterium flavum

Yeast sludge was used as a substrate in growth medium of *B. flavum* for the production of lysine. Different substrate (yeast sludge) to water ratios (10 mL yeast sludge: 90 mL H_2O, 20 mL yeast sludge:80 mL H_2O, 30 mL yeast sludge : 70 mL H_2O, 40 mL yeast sludge: 60 mL H_2O , 50 mL yeast sludge : 50mL H_2O, 60 mL yeast sludge: 40 mL H_2O, 70mL yeast sludge: 30 mL H_2O, 80 mL yeast sludge : 20mL H_2O, 90 mL yeast sludge: 10 mL H_2O and 100 mL yeast sludge as such) were tested for lysine production. Triplicate flasks containing 100 mL of growth medium were incubated for 2 days at pH 7 and temperature 35°C. The biomass was homogenized and suspension was then analyzed for lysine. Optimum substrate (yeast sludge) concentration was adopted in subsequent experiments. Effect of various time periods on lysine production from *B. flavum* was investigated. To examine the effect of temperature, the fermentation test was performed at various temperatures (30, 35, 40, 45, 50 and 55 °C) at pH 7; to examine the effect of pH, the experiment was carried out at various pHs (5, 5.5, 6.0, 6.5, 7.0, 7.5, 8) at 35 °C.

Effect of Molasses, Corn Steep Liquor and Protein Hydrolysate on Lysine Production from B. flavum

Molasses, corn steep liquor and protein hydrolysate of sesame meal were optimized in the fermentation medium to increase the lysine production from *B. flavum.*

Proximate Analysis of the Yeast Sludge

Proximate analysis of the yeast sludge was performed before and after fermentation with *B. flavum* following A.O.C Methods [29] 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* according to the method described by Moore and Stein [38].

Statistical Analysis

The data obtained was subjected to Analysis of Variance Techniques [39] and, in case of significant differences, a DMR was applied [40].

Conclusion

The fermentation conditions for the microbial production of lysine by *B. flavum* were

successfully optimized on laboratory scale. Maximum lysine concentration was achieved with 40 % substrate (yeast sludge) concentration, 1 % molasses, 4 % corn steep liquor and 25 mg % protein hydrolysate of sesame meal at pH 7, 35 °C at 48 h. Fermentation of yeast sludge with *B. flavum* increased the lysine content from 1.54 % to 4.78 %. The lysine enriched biomass obtained from fermentation of yeast sludge has the potential for usage in poultry and livestock production.

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