REPORT

Bioconversion of agriculture waste to lysine with UV mutated strain of *brevibacterium flavum* and its biological evaluation in broiler chicks

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Abstract: Lysine executes imperative structural and functional roles in body and its supplementation in diet beneficial to prevent the escalating threat of protein deficiency. The physical mutagenesis offers new fascinating avenues of research for overproduction of lysine through surplus carbohydrate containing agriculture waste especially in developing countries. The current study was aimed to investigate the potential of UV mutated strain of *Brevibacterium flavum* at 254 nm for lysine production. The physical and nutritional parameters were optimized and maximum lysine production was observed with molasses (4% substrate water ratio). Moreover, supplementation of culture medium with metal cations (i.e. 0.4% $CaSO_4$, 0.3% NaCl, 0.3% KH_2PO_4, 0.4% $MgSO_4$, and 0.2% $(NH_4)_2SO_4$ w/v) together with 0.75% v/v corn steep liquor significantly inhanced the lysine production up to 26.71±0.31g/L. Though, concentrations of urea, ammonium nitrate and yeast sludge did not exhibit any profound effect on lysine production. Biological evaluation of lysine enriched biomass in terms of weight gain and feed conversion ratio reflected non-significant difference for experimental and control (+ve) groups. Conclusively, lysine produced in the form of biomass was compatible to market lysine in its effectiveness and have potential to utilize at commercial scale.

Keywords: Lysine, *Brevibacterium flavum,* UV mutation, agriculture waste, biological evaluation.

INTRODUCTION

Lysine is one of the essential amino acid required to perform various physiological functions in human body (Cheng and Zhang, 2007). In developing countries people are consuming cereal-based foodstuffs that are deficient in lysine so, it is required to enhance the protein intake to evade the escalating threat of protein deficiency (Millward, 2012). Therefore, it is the need of time to supplement existing diet with lysine to meet the nutritional requirements for human health and domestic meat producing animals including poultry and fisheries (Moosavi-Nasab *et al.,* 2007).

Specifically, in the poultry industry utilization of vegetables and plant based ingredients for formulation of poultry feed lacks amino acids as, plant proteins are deficient in essential amino acids especially lysine (Han *et al.,* 1992; Bera *et al.,* 2010). Low crude protein broiler diets supplemented with the limiting amino acids is economic in production but may leads to the deficiency of essential nutrients to animal as well as human diet (Labadan *et al.,* 2001; Si *et al.,* 2004).

Presently, amino acid demand is continuously increasing all over the world due to its broad utilization/applications in pharmaceutical industry as well as the precursors for the synthesis of peptides (Tryfona and Bustard, 2005). Lysine is produced in considerable amount across the world by fermentation process using carbohydrates as substrate and mutant strains of *Corynebacterium* and *Brevibacterium* species (Shah and Khan, 2008; Bera *et al*., 2010). Consequently, it would be imperative to increase lysine production by utilizing all the available cheap and non-conventional lysine-yielding resources (agriculture and industrial waste) (Moosavi-Nasab *et al.,* 2007).

The capability of *Corynebacterium* and *Brevibacterium* to produce amino acids was documented in 1950s. Beside these organisms, recombinant *E. coli* strains are used as alternate source of lysine production (Wittmann and Becker, 2007). The non-pathogenic species of Coryneform bacteria including *Corynebacterium glutamicum, Brevibacterium lactofermentum,* and *Brevibacterium flavum* used to produce amino acids via

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fermentation (Hirasawa *et al.,* 2000; Rehman *et al.,* 2012). Two Coryneform species (*B. flavum* and *C. glutamicum*) are broadly utilized for lysine and glutamic acid production on industrial scale (Xu *et al.,* 2010). Superfluous lysine can be synthesized through mutated *Brevibacterium flavum* by exposure of Ultraviolet (UV) radiations (Naz *et al.,* 2001; Rehman *et al.,* 2012). In this study *Brevibacterium flavum* was used for lysine production via UV exposure. The current study was aimed to investigate the potential of UV mutated strain of *Brevibacterium flavum* at 254 nm for lysine production from agricultural wastes and to estimate this production via biological evaluation processes.

MATERIAL AND METHODS

Microorganisms and culture conditions

Brevibacterium flavum bacterial strain was obtained from Department of Biochemistry, Agriculture University Faisalabad. Nutrient agar was used to culture the strain on at 30°C and organism gram staining, catalase and gelatin liquefaction tests were performed to identify the character and purification of organism (Mehmood, 1996).

Formation of UV mutants of Brevibacterium flavum

Formation of UV mutants of *Brevibacterium flavum* was executed according to the Atef *et al,* (2007) and Naz *et al,* (2001), for this purpose sterile liquid medium was inoculated with the *Brevibacterium flavum* and shacked for 24h at 30°C. Afterward, 1mL was taken as control and rest of the medium was used for the exposure of UV radiations (254nm) at different time intervals 5, 10, 15, 20, 25 and 30min. Then, six agar media plates were streaked with different inoculums exposed with UV radiations to check growth of separate colonies. Single colony of mutated *Brevibacterium flavum* was taken and transferred to sterilized inoculum medium flask and placed on orbital shaker for 48h at 120rpm until the achievement of optical density i.e. 0.6 at 600nm (Naz *et al.,* 2001).

The proximate analysis of substrates

The proximate analysis (crude fat, crude protein, ash content, crude fiber and nitrogen free extract) of molasses, wheat-bran, and rice polishing was carried out according to AOAC, (1990) methods.

Production and estimation of lysine

Fermentation media was prepared for lysine production by adding components enlisted in table 1(Moosavi-Nasab *et al.,* 2007). Spectrophotometric method was used for the estimation of lysine (Chaves *et al.,* 1988). The standard solution of lysine was prepared and treated with acetone, sodium nitroprusside and sodium tetraborate. Subsequently, test tubes were shacked for 60 min. and volume adjusted as 20mL with distilled water. The absorbance of each standard solution was checked at 545 nm on spectrophotometer. Samples were also treated in the same way as standard solutions.

Optimization of different parameters for fermentation

The below stated conditions were optimized for lysine production by mutated strain of *Brevibacterium flavum* (Sattar *et al.,* 2008).

Substrate water ratio with incubation period

Molasses, wheat bran, and rice polishing were subjected to fermentation process along with basal fermentation medium at different substrate water ratio (1 to 5%) for different incubation periods (24h to 72h). A total 45 flasks were subjected to fermentation in order to check optimum substrate water ratio and incubation period to select one substrate for lysine production.

Effect of temperature on lysine production

Fermentation experiment was repeated with optimized substrate and substrate water ratio along with optimized incubation period adjusted at different temperatures 25, 30, 35, 40 and 45°C at 120 rpm.

Effect of salt concentration

After optimization of substrate water ratio, incubation period and temperature; fermentation experiment was repeated with different percentages (0.1, 0.2, 0.3, 0.4 and 0.5%) of calcium sulphate ($CaSO_4$), magnesium sulphate ($MgSO_4$), sodium chloride (NaCl) and potassium dihydrogen phosphate (KH_2PO_4) to determine the optimum concentration of salt for maximum production of lysine.

Effect of different nitrogen sources

In order to check lysine production with different concentrations of organic and inorganic nitrogen sources the fermentation process was started with all optimized conditions. The flasks having concentrations of ammonium sulphtae $(NH_4)_2SO_4$, urea and ammonium nitrate (NH_4NO_3) ranged between ranged 0.2% to 1% and 0.25% to 1.25%, respectively, were placed on orbital shaker to Proceed fermentation.

Addition of corn steep liquor

After the ionic salts and nitrogen sources optimization, the fermentation process was repeated with the different concentrations (0.25, 0.5, 0.75, 0.1 and 1.25%) of corn steep liquor to check the production of lysine as, it is considered best source of protein as well as an organic nitrogen source.

Production of lysine at large scale at pre-optimized conditions

After the optimization of all the parameters fermentation at large scale was carried out in 10L flask by using UV mutants of *Brevibacterium flavum* and biomass obtained was supplemented to broiler chick's diet according to percentage of additional lysine in commercial feed of

poultry. Before addition, the lysine containing biomass was concentrated in water bath at 60-70^{o}C in order to make easier to mix in poultry feed.

Biological evaluation

Day-old broiler Chicks (n=80) and their feed were procured from Big birds hatchery and fed on basal diet for seven days to acclimatize the standard laboratory conditions before study. At the end of first week sixty broiler chicks (n=60) were selected on uniform weight basis under completely randomized design (CRD) and divided into three groups: Group 1 (*n*=20) was considered as control and feed on commercial diet while, Group 2 (*n*=20) was considered as experimental group in which diet with same concentration of lysine as in commercial diet (0.6%) was supplemented in the form of biomass produced. On the other hand a third group (*n*=20) was considered as negative control group, which was fed on lysine excluded commercial diet. The room temperature of the chicks was maintained at 95°F in the first week. Thereafter temperature was decreased by 5°F after every week until 75°F. Feed & water were offered on *adlibitum* basis during the five weeks feeding trial. Vaccination was done after 6 and 20 days for New Castle disease and after 11 and 25 days against Infectious Bursal disease. The body weights of each group were recorded at the start of experiment then weekly. Biological evaluation of the diets fed to broiler chicks were evaluated in terms of weight gain (gm), feed consumption (gm) and feed conversion ratio (FCR) (Saima *et al.,* 2010)

STATISTICAL ANALYSIS

Basic descriptive statistical analysis and one-way ANOVA (P>0.05) were performed using the Statistica software version 5.5 for the biological evaluation of data. Costat-2003, Co. Hort (version 6.303) software was used for comparison of data with Duncan's multiple range and LSD.

RESULTS

Proximate analysis

The proximate analyses of various substrates such as rice polishing, wheat bran and molasses were executed to analyze the composition specifically moisture, ash, ether extract, crude protein, crude fiber and nitrogen free extract (NFE). The results obtained are summarized in table 2.

Microscopic identification of microorganism

The number of colonies of wild strain *Brevibacterium flavum* was subjected to UV radiations for different time exposures (5, 10, 15, 20 and 25 min) and complete killing of organisms was observed at 15 min. Consequently, experiment was repeated at time interval (2, 4, 6, 8, 10, 12, 14 and 16 min) with UV exposure from15 cm distance and maximum death of organism was observed at 16 min. while minimum growth was observed at 10 and 12 min. However, the maximum lysine production was recorded with mutant exposed for 12 minutes.

Effect of substrate water ratio of agriculture waste

The effect of various substrate water ratios 1, 2, 3, 4 and 5% (w/v) of molasses, rice polishing and wheat bran on production of lysine was assessed at different incubation periods (24h, 48h and 72h). The utmost lysine production was observed (11.79±0.15g/L) at 4% substrate water ratio with molasses at 72h incubation period as compared to wheat bran and rice polishing (fig. 1). Based on statistical analysis, molasses was selected among all the substrate to continue fermentation for the bioconversion of agriculture waste to lysine because of maximum yield.

Effect of temperature

The effect of temperature on lysine production consuming molasses as substrate at 72h incubation period was determined and revealed that lysine production was increased gradually with the increase in temperature up to 30°C. Low yield was observed at 15°C and 35°C (fig. 2).

Effect of different salts and nitrogen sources

The experimental results regarding optimization of different ionic salts concentration at 30°C with 4% molasses for 72h revealed that growth media containing 0.4% of $CaSO_4$ facilitated maximum lysine production i.e. 15.04g/L (table 3). Likewise, different concentrations of sodium chloride (NaCl) and potassium di-hydrogen phosphate (KH_2PO_4) were evaluated for efficiency to enhance lysine production with all previous optimized conditions. The highest yield (16.62g/L and 16.74g/L) was achieved at 0.3% NaCl and 0.3% KH_2PO_4, respectively (table 3). However, the maximum yield was attained at 0.3% KH_2PO_4, but results revealed non-significant difference from yield at 0.2%. Moreover, $MgSO_4$ was also evaluated for its efficiency to enhance lysine production with all previous optimized conditions and the highest yield (20.01±0.23g/L) was achieved at 0.4% $MgSO_4$ (table 3).

The optimum concentration of ammonium sulphate $(NH_4)_2SO_4$ was evaluated in the fermentation process and the highest lysine yield (20.4±0.36g/L) was observed at 0.2% w/v (table 4). The statistical analysis for $(NH_4)_2SO_4$ revealed that lysine production at all concentrations was highly significant. Yeast sludge was considered as lysine deficient protein source, having anti-aflatoxin effect in Broiler chicks. In the present study, different concentrations of the yeast sludge with all previous optimized conditions did not demonstrated any profound effect on lysine yield (table 4). The highest production of lysine (26.71±0.31g/L) was found at 0.75% v/v CSL.

Large scale production of lysine and its biological evaluation

The equal amount of feed with equal level of crude protein was provided to each group of broiler chicks including positive control, negative control and experimental (mutant) group on *adlibitim* basis and at the end total feed intake was computed almost 74Kg for each group (average 3.675Kg per chick). The data obtained was statistically analyzed and lysine level exhibited significant effect on weight gain and feed conversion ratio (FCR). The outcomes revealed that the lowest and the highest value of weight gain intended for all feed treatments were perceived during the first and fourth week, respectively. Moreover, during the fourth week of treatment, the analysis of variance directed that experimental group and control (+ve) group were non-significant ($P \leq 0.05$) from each other however, both were significantly different with respect to control (-ve) group (fig. 3A).

Feed conversion ratio was calculated by dividing feed consumption on weight gain and thus data obtained was statistically analyzed. In comparison to all weeks, the overall utmost feed consumption ratio (FCR) for all feed

Table 1: Composition of basal fermentation medium

Ingredients	Amount (g/L)	Salts	Amount (g/L)
$MgSO_4$	1	$MnSO_4$	0.016
KH_2PO_4	2	$CaCO_3$	2
K_2HPO_4	2	$(NH_4)_2SO_4$	5
Vitamin B Complex (Soln.)	2-3 *(drops)*	Urea	5
		Biotin	0.06

Table 2: Proximate composition of the substrates used for production of lysine

Composition (%)	Rice polishing	Wheat bran	Molasses
Moisture	7.30	8.50	18.9
Ash	12.4	5.72	7.98
Ether-extract	15.0	4.00	---
Crude protein	12.2	14.7	2.80
Crude fiber	4.00	11.65	---
Nitrogen free extract	48.5	53.23	70.62

Table 3: Effect of various concentrations of salts (Calcium Sulphate, Potassium di-Hydrogen Phosphate, Sodium Chloride and Magnesium Sulphate) on lysine production (g/L) by *Brevibacterium flavum*

Salts	Concentration (g/100mL)				
	0.1	0.2	0.3	0.4	0.5
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
$CaSO_4$	6.4±0.29e	8.05±0.31d	11.1±0.24b	15.04±0.29a	10.35±0.2c
KH_2PO_4	16.11±0.28b	16.46±0.26ab	16.74±0.37a	13.49±0.26c	7.57±0.21d
NaCl	13.39±0.35c	15.51±0.29b	16.62±0.27a	11.59±0.37d	8.63±0.26e
$MgSO_4$	15.23±0.31d	16.54±0.29c	17.54±0.32b	20.01±0.23a	14.82±0.18d

Table 4: Effect of various concentrations of nutritional parameters (Urea, Ammonium Nitrate, Corn Steep Liquor, Ammonium Sulphate, and Yeast Sludge) on lysine production (g/L) by *Brevibacterium flavum*

Salts	Concentration (g/100mL)				
	0.25	0.50	0.75	1.00	1.25
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Urea	10.15±0.32a	8.54±0.35b	5.13±0.24c	4.33±0.18d	2.82±0.21e
Ammonium Nitrate	3.87±0.26c	6.89±0.15a	6.09±0.19b	5.78±0.22b	1.63±0.27d
Corn Steep Liquor	22.84±0.18c	24.61±0.27b	26.71±0.31a	24.26±0.34b	18.06±0.26d
SALTS	0.2	0.4	0.6	0.8	1.0
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Ammonium Sulphate	20.4±0.36a	17.3±0.32b	15.23±0.22c	12.84±0.18d	8.37±0.20e
Yeast Sludge	0.64±0.03a	0.55±0.03b	0.42±0.02c	0.27±0.1d	0.17±0.07e

Means sharing the same letter in a row (mineral) are significantly same.

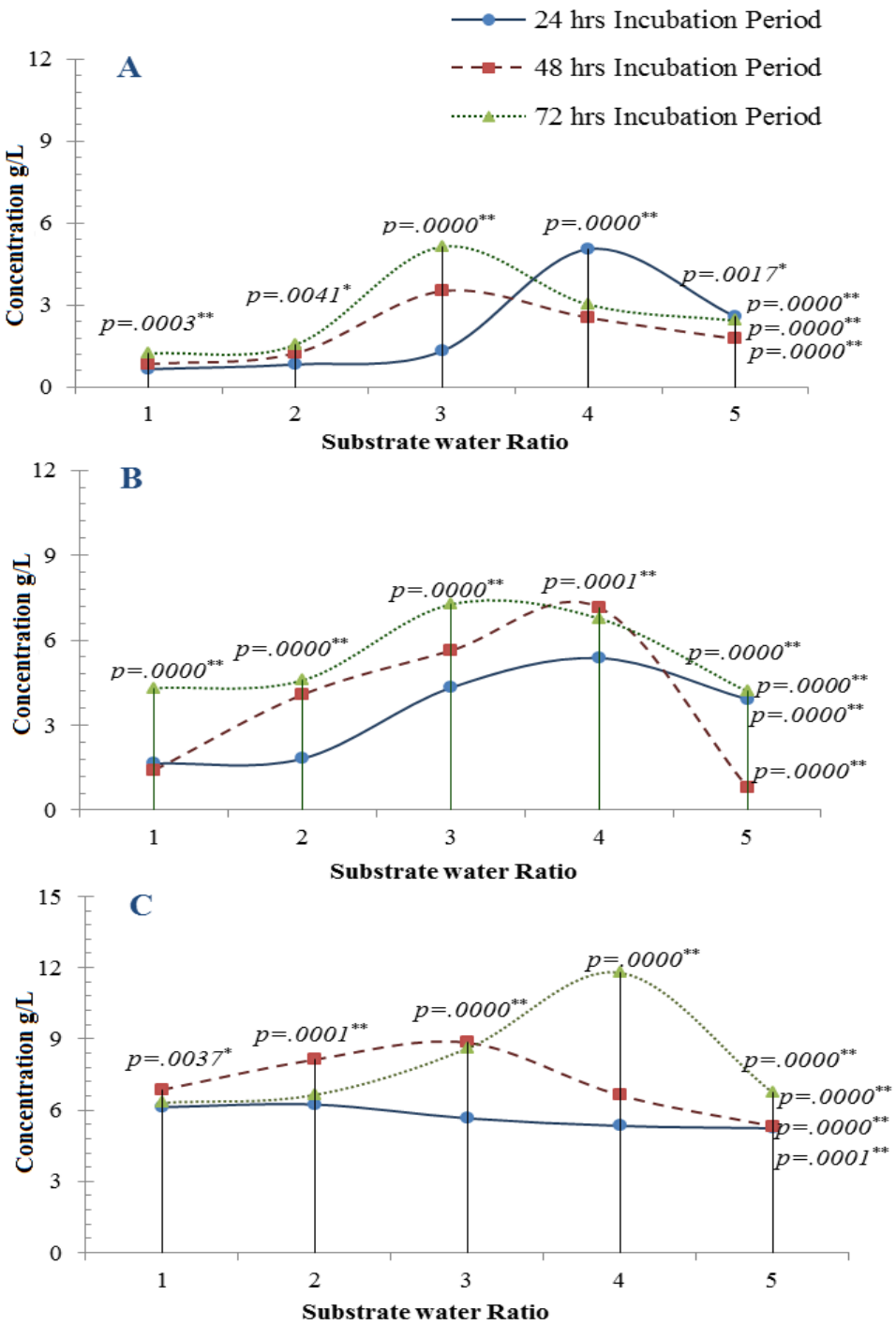

Fig. 1: Effect of incubation period and substrate water ratio of (**A**) rice polishing (**B**) wheat bran (**C**) molasses on lysine production (g/L)

treatments was observed during the second week whereas; minimum FCR was observed during third week of experiment. Results for first and second week demonstrated that all treatments were significantly different from each other but during third week of experiment, non-significant results were observed for all treatments. During fourth week of treatment results indicates that lysine experimental group and control (+ve) were statistically non-significant but both were significant from control (-ve) group. The overall outcome appeared same in fourth week (fig. 3B).

DISCUSSION

Aim of the present study was to investigate the increase in production of lysine by utilization of agriculture waste

and its biological evaluation in broiler chicks for muscle development as it was hypothesized that it is effective for the enhancement of muscles in Broiler chicks. The study was conducted in a comprehensive way, however, for better understanding of the readers, results and their interpretations are divided into two major parts i.e. production of lysine at optimized conditions and then on pilot scale, thereafter its biological evaluation was carried out by conducting a feeding trial in broiler chick.

The results of present study are consistent with Naz *et al.,* (2001) who performed trial on *Brevibacterium flavum* for lysine synthesis and revealed 4% molasses as optimized concentration. Similarly, results regarding incubation periods are closely compatible with Rehman *et al.,* (2012) who optimized incubation period as 60h for lysine production that is approaching to 72h. The same outcomes concerning temperature optimization are in accordance with findings of Pasha *et al.,* (2011) and Shah *et al.,* (2002a) about maximum lysine production from *Brevibacterium flavum* at 30°C.

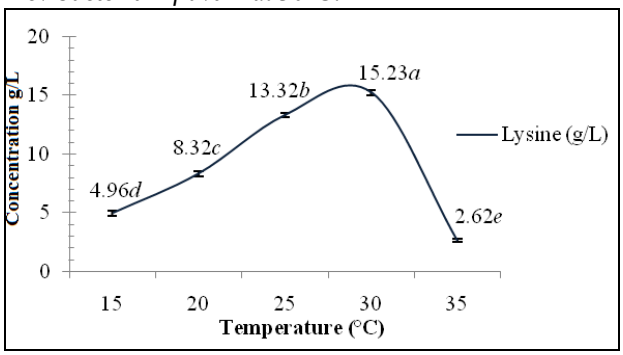

Fig. 2: Effect of various temperatures on lysine production (g/L) by *Brevibacterium flavum*

Supplementation of culture medium with metal cation improved substantially the lysine production through *B. flavum*, exhibiting the over-production with calcium, magnesium, sodium and potassium salts. Similar concentration of $CaSO_4$ was consumed by Ali (2004) to attain utmost yield of lysine indicating in accordance with current analysis.

Sodium chloride is common metallic salt that plays an important role in membrane transport system in prokaryotes and eukaryotes (Suenaga *et al.,* 2006). Correspondingly, KH_2PO_4 was considered as good source of phosphorous required for ATP synthesis (Mehmood 1996). Microbial production of lysine with auxotrophic mutant strain of *Corynebacterium glutamicum* by Shah *et al.,* (2002a) exhibited results regarding NaCl concentration consistent with current findings and reported better results as compared to $MgCl_2$ utilization. In another study, higher pyhtase production by halophilic pseudomonas isolated from fish intestine observed at 3% optimized NaCl concentration (Esakkiraj *et al.,* 2010). It might be assumed that higher concentration of NaCl probably instigate reverse osmosis that was responsible for death of fermenting organism. In other studies, beneficial influences of metal salt $MgSO_4$ with 0.2% concentration were observed for the production of lysine and tannase (Ali 2004; Shah *et al.*, 2002a,b).

Nitrogen is one of the major elements of amino acids and its sufficient concentration from suitable source is required for overproduction of lysine. The nitrogen source such as ammonium sulphate and ammonium chloride are assumable and facilitate the cellular growth accompanied by product formation and their required amount should be added in culture medium (Ali *et al*., 2009).

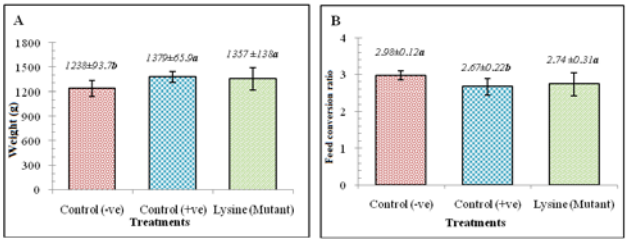

Fig. 3: Mean ± SD of body weight gain (A) and feed conversion ratio (B) for various lysine treatments during 4 weeks of biological trial on broiler chicks

The same optimum condition like in this study was reported by Shah *et al.* (2002a,b) who depicted a better production of lysine with almost 2% $(NH_4)_2SO_4$. Similarly, the effect of other nitrogen sources such as ammonium nitrate and urea (table 4) was evaluated to enhance lysine production, but indistinct outcomes were perceived. Lessened yield caused by urea treatment attributed to some lethal effects of urea that contributed to interrupt the lysine production (self-perception). Impede lysine production can be explained as the yeast cells revived to compete with fermenting organism by the consumption of nutrients in fermentation media (Cui *et al.,* 2012).

Corn steep liquor (CSL), another organic nitrogen source has shown a prominent effect on lysine production (table 4). The present results are in accordance with Naz *et al.,* (2001), who utilized CSL as nitrogen source for the production of lysine by UV mutants of *Brevibacterium flavum*. Naz *et al.* (2001) suggested less than 1% CSL for maximum yield of lysine as production was continuously decreasing from 1-4% in their findings, moreover same type of results were also reported by Mehmood, (1996).

Large-scale production was executed after the optimization of each nutrient with the intention to acquire lysine-comprising biomass. This biomass was employed to supplement the broiler chick's diet as replacer of commercial lysine and it was analyzed for its biological efficiency. During the biological trial, weight gain, feed consumption, and feed conversion ratio were calculated to evaluate the effect of lysine supplemented in the form of biomass.

The consequence of this trial correlates with the Sterling *et al.*,(2003) who reported that the reduction in dietary CP level and supplementation of lysine leads toward high feed consumption as compared to the high CP diet with normal lysine levels. However, the results fluctuated because in present study feed was provided on *adlibitum* basis (never left the feeder empty) to all groups. Saima *et al.,* (2010) was also documented non-significant difference in feed conversion ratio among the groups provided with lysine-supplemented diet. The variance in results because of different experimental design as in their study different percentage of lysine was applied from one source. Whereas, in present study, lysine from two different sources was used with the same concentration and a negative control group (without lysine) was also allotted.

Lysine is essential ingredient of diet but other amino acids are also required. Whereas, broilers require high lysine for weight gain and feed efficiency (Labadan *et al.,* 2001). In various studies, it is indicated that lysine is required in higher concentration at early stage of growth, which enhanced the weight gain and feed conversion ratio (Labadan *et al.,* 2001; Si *et al.,* 2001). The results of Almeida *et al.,* (2002) supported the present findings by expressing that the breast meat development of broilers is directly proportional with higher lysine levels in diet. Likewise, Saima *et al.,* (2010) evaluated the effect of different concentration of lysine supplementations on broiler chicks and reported 1244g as maximum weight gain while, comparatively in present study the maximum weight gain was observed 1357g at the end of experiment.

The findings of present study were an agreement with the previously published report (Saima *et al.,* 2010) which indicated non-significant difference in FCR of the groups treated with lysine-supplemented diet however, in that study different percentage of lysine was applied from one source whereas, the current study, lysine from two different sources was applied through the same concentration with negative control group (without lysine).

CONCLUSION

The current study reflected that supplementation of lysine containing biomass contributed to improve weight of the broiler chicks. Overproduction of lysine by mutated *Brevibacterium flavum* through fermentation of non-conventional resources like molasses was investigated that should be beneficial in term of economy and nutritional supplements. Practice of lysine production through mutant bacterial strains will help to reduce the cost of commercially available lysine.

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