

Bioconversion of Agricultural By-Products to Lysine by *Brevibacterium flavum* and Physico-Chemical Optimization for Hyper-production

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Summary: Poultry and agriculture industry has a great role in the development of food sector in Pakistan. Whole of the Lysine required for poultry feed is imported to fulfil the desired dietary needs. Present study was designed to utilize different agricultural by-products like molasses, wheat bran, rice polishing and corn steep liquor. Different Physico-Chemical parameters were optimized to have hyper-production of Lysine through fermentation by using *Brevibacterium flavum* as a fermentative agent. From wheat bran, rice polishing and molasses (as best carbon source), significantly high concentrations of lysine (10.4 g/L) after 72h of incubation was observed with molasses (4%) with 3% (v/v) inoculum size at 30°C and pH 7. Among different nitrogen sources, 0.25% (NH₄)₂SO₄ showed significantly (P< 0.05) high yield of Lysine (16.89 g/L). Addition of different optimum levels of ionic salts; 4% CaCO₃, 0.4% MgSO₄.7H₂O, 0.1% NaCl and 0.2% KH₂PO₄ gave significantly (P< 0.05) higher quantity of Lysine 19.01 g/L. Inclusion of 0.6% corn steep liquor and 0.4 mg/100mL biotin significantly (P< 0.05) raised the Lysine from 19.4 g/L– 19.45 g/L. The presence of Lysine in fermented broth was detected by TLC. Thus a cheap and practical bioprocess of Lysine production was concluded, that can be exploited commercially to save foreign exchange.

Key words: Agricultural by-products; *Brevibacterium flavum*; Lysine; molasses; Wheat bran; Rice polishing; Fermentation

Introduction

Demand for good quality protein food is increasing day by day for human and animals. Hence constant efforts are required to enhance the productive activity through conventional and non-conventional processes [1]. Feed sector has always been in need of economical and quality protein sources [2]. As Pakistan is an agricultural country many agricultural and industrial by-products like molasses (cane, beet), starch waste, rice polishing, wheat-bran and corn steep liquor are abundantly available. These by-products serve as potential source of energy for production of low cost, high quality microbial biomass proteins and amino acids. The microbial biomass protein can be fed to poultry and human beings. Molasses are valuable by-product of sugar industry and a good source of carbon and minerals. Pakistan sugar sector has the capacity to produce over 2.5 million metric tons of molasses [3] and this huge source of industrial by product can be utilized for lysine production.

Lysine, one of the most important and critical essential amino acids, and its demand has been steadily increased in recent years. It is the

second most produced amino acid on a large industrial scale, more than 800,000 tons per year [4]. Over 40,000 tons of lysine is utilized per year mainly in nutritional supplements and animal feed [5]. In the year 2009, lysine production for the animal feed reached 700,000 tons. It is therefore imperative to produce lysine through fermentation of agricultural by-products which will be used to produce high quality protein in terms of meat and milk. Lysine helps in the absorption of calcium so it prevents from osteoporosis and is needed for the bone formation of children and growing animals [6]. Lysine plays an important role in production of antibodies for healthy immune system. Lysine has an important role in body building and helps in speed recovery of damage muscles caused by over fatigue [7].

The microbial process of fermentation is currently gaining ground because it has produced the high cost compounds from the low cost industrial and agricultural by- products. The lysine is being produced on industrial scale using *Coryneform* bacteria as fermenting agent [8, 9]. *Brevibacterium flavum* was found to be the best fermenting agent that

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was used in this study for lysine production. Pakistan feed industry imports all the required lysine for the production of about 542.74 millions of broilers, and only 0.1- 0.4% lysine are added to broiler feed [10], this shows that this minimum requirement of lysine could be fulfilled by locally produced lysine through fermentation of agricultural by- products. In this study, agricultural by- products were used as substrate and lysine production was optimized with respect to substrate water ratio, incubation period, temperature, ionic concentrations, organic and inorganic nitrogen source using *Brevibacterium flavum*.

Experimental

Bacterial strain and maintenance

Brevibacterium flavum (NRC- 207 F Rev. 2/78) was procured from the International University Tokoyo, Japan. The organism was revived and maintained on nutrient agar plates (pH 7± 0.2).

Physico-Chemical Optimization for Hyper-Production of Lysine

Seed culture medium was prepared by following Nasab [20] and one full loop from refreshed slant culture was inoculated. Culture 18 hours old, having OD (0.6) at 660 nm was used as inoculum [24]. Different agricultural by-products like cane molasses, wheat bran and rice polishing (1, 2, 3, 4 and 5 %) were used for optimization of the best carbon source. Various degrees (25, 30, 35, 40°C) of temperature were optimized to yield maximum Lysine by using the pre-optimized carbon source as a substrate. Different percentages (1, 2, 3 and 4) of inoculum were used to optimize the size of inoculum to get high titre of Lysine [19].

Different concentrations (0.25, 0.5, 0.75, 1.0 and 1.25%) of NH_4NO_3 , urea and $(\text{NH}_4)_2\text{SO}_4$ were taken in basic fermentation medium to optimize the best nitrogen source [20]. Corn steep liquor with different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 % v/v) was used to get the optimized concentration [24].

Various concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 %) of ionic salts like $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NaCl and KH_2PO_4 were used for optimization. The concentrations (1, 2, 3, 4 and 5 %) of CaCO_3 were optimized to get high yield of Lysine. It was dry sterilized with respect to other medium and added later with sterilized conditions [10]. Biotin concentration (0.1, 0.2, 0.3 and 0.4 mg/100mL) were used to enhance Lysine contents [28]. All the parameters were optimized in shake flask of 250 mL

(Erlenmeyer flask) having 25 mL total fermentation medium.

Fermentation

The basal medium for fermentation was prepared by adding optimized concentrations such as molasses (40 g), $(\text{NH}_4)_2\text{SO}_4$ (2.5 g), urea (5 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (4 g), $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (2 g), NaCl (1g), CaCO_3 (40 g), corn steep liquor (6 ml), biotin (4 mg), vitamin B complex (2-3 ml) per litre. The pH was adjusted at 7± 0.2 with 1N NaOH and media was autoclaved for 15 minutes. The fermentation was carried out on orbital shaker at 180 rpm at 30°C for 72h [20].

Lysine Estimation

The fermented broth was autoclaved and centrifuged at 10,000 rpm for 10 minutes. Then the supernatant was used for both qualitative and quantitative estimation of lysine produced [31]. Qualitative estimation of lysine in broth was carried out through TLC, by using CHCl_3 , CH_3OH and 17% NH_3 solution (2:2:1) as a solvent [35]. Sample (5µl) was spotted on pre-coated TLC plate and kept for 4-6h at 4°C in the chromatographic tank. Then dried plate was sprayed with 0.2% ninhydrin solution in acetone and heated the plate at 110°C for 5 min in preheated oven. The lysine produced in the fermented broth was quantitatively measured by spectrophotometric method reported by Chaves [32].

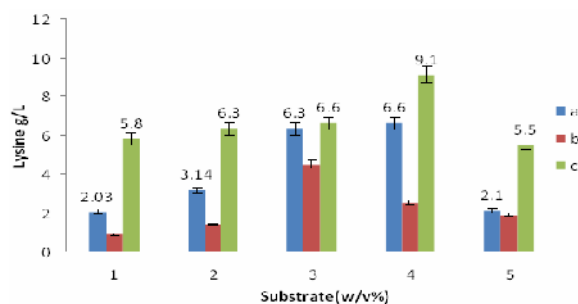
Statistical Analysis

All the experiments were performed in triplicates. The data was analysed on SPSS 13.0 software, by compare mean through One-Way ANOVA and multiple comparison was made through LSD and Descriptive analysis.

Results and Discussion

As Pakistan is an agricultural land and hundred thousand tons of agricultural by-products are produced annually that could be utilized for Biotechnological production of Lysine. Therefore, different agricultural by-products like wheat bran, rice polishing and molasses were tested to have an inexpensive source of carbon for the production of Lysine with respect to time. Wheat bran (4%) gave the maximum yield (6.6 g/L) of Lysine after 72h of incubation. While 3% rice polishing and 4% molasses after 72h of incubation period was found optimal for maximum Lysine production at pH 7 by using *Brevibacterium flavum* as fermenting agent (Fig. 1), if the concentration of these were increased beyond the optimal level reduction in the lysine concentration was observed. By comparing these three carbon sources, molasses yielded the highest

Lysine (9.1 g/L) therefore it was used as a substrate for further optimization. These results are in line with Khan [11], who also found molasses as the best agricultural by-product for the production of essential amino acid Lysine through fermentation by *Corynebacterium glutamicum*. The observations of Naz [12] have also supported the present results. They investigated the Lysine production with 4% molasses at pH 7 and 37°C by *Brevibacterium flavum* and recorded lysine titre 2.1 per cent. Rao [13] reported 72h as a best fermentative period for lysine production (45.34 g/L) at 30°C and pH 7.5 which is also in agreement with present findings. Whereas, Sattar [14] reported the enhancement of lysine concentration from 1.54%- 4.78% after 48h of incubation through fermentation of yeast sludge. Among all of the checked agricultural by-products as a substrate, molasses was found a cheap carbon source. This contains 40-50% sucrose that is utilized by the organism for the maximum production of lysine.

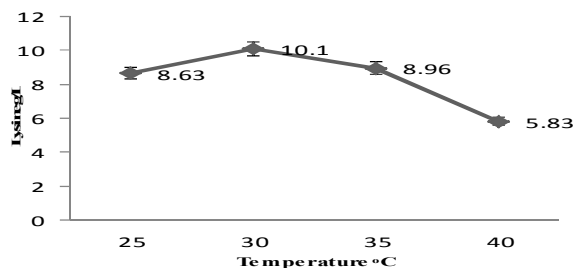


(a) wheat bran (b) rice polishing (c) molasses. Error bars show the increasing percentage of Lysine with respect to different % of substrates

Fig. 1: Optimization of different substrates (%) for Lysine production.

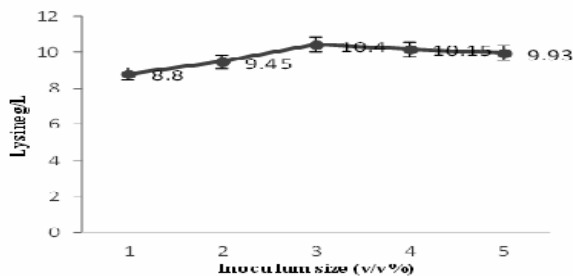
Metabolic activity of an organism is greatly affected by change of temperature. Therefore to optimize a suitable temperature, fermentation was carried out with 4% molasses as substrate for 72h of incubation at various degrees of temperature. The results showed that lysine production was significantly ($P < 0.05$) higher at 30°C as compared to all other temperatures. Further increase in temperature resulted in decline of lysine titre (Fig. 2a). The results of present study are in line with the work of Sthiannopkao [15], who also observed 30°C as optimum temperature in their study while working on three strains of *Coryneform* bacteria during fermentation. Nakamaya [16] cultured *Brevibacterium* and *Corynebacterium* auxotrophs on 20-40°C and reported maximum lysine production 17- 39g/L at 30°C that is also in support of our

optimized temperature. The results of Shah [5]; Nelofer [17]; Rao [13] showed that at 30°C, the metabolism of *Coryneform* bacteria are highly active for the production of enzymes and amino acids. Cultivation temperature used by Javaid [18] was 32°C with mutated strain of *Brevibacterium flavum*, using glucose (3%) and inoculum size of 8% at 7.5 pH. They reported lysine production 8.8g/L during fermentation in shake flask (25mL) and 17.8g/L in 7.5 L fermenter. Naz [12] reported maximum lysine (21.48 g/L) production by *Brevibacterium flavum* at 37°C that was in contrast to present findings. The amount of required product produced through fermentation is also greatly affected by the size of inoculum. Inoculum size of 3% showed significantly ($P < 0.05$) high yield 10.4 g/L of lysine (Fig. 2b). Adnan [19] found 4% inoculum size as optimum level and reported maximum production of lysine 23.57 g/L by using *Brevibacterium linens*. Whereas Javaid [18] investigated 8% inoculum size as optimum level of *Brevibacterium flavum* (IIBUV2) to get the maximum lysine production.



Error bars show the different percentages of Lysine produced at different temperatures

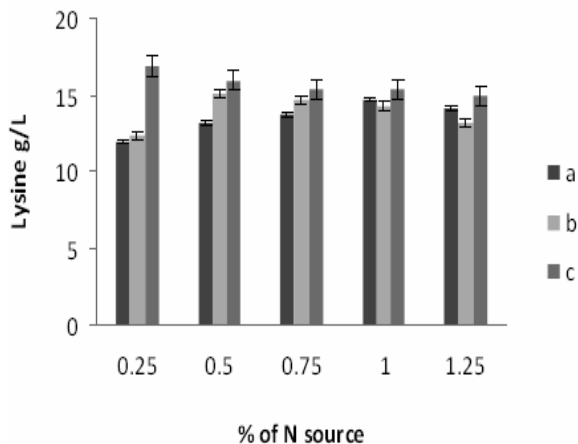
Fig. 2 (a): Effect of temperature on the production of Lysine through fermentation with 4% molasses by *Brevibacterium flavum*.



Error bars show the different percentages of Lysine produced with different inoculum sizes

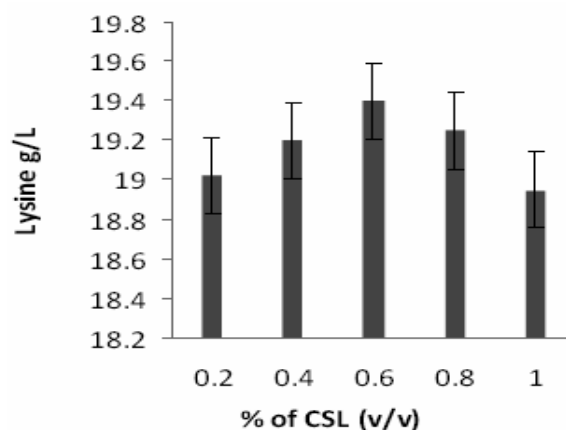
Fig. 2(b): Effect of inoculum size on the production of Lysine through fermentation with 4% molasses by *Brevibacterium flavum*.

It was reported [20], that coryneform bacteria need ample supply of nitrogen as Lysine contains 19.16% nitrogen. Therefore effect of different organic and inorganic nitrogen sources like ammonium nitrate (NH_4NO_3), urea and ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$) was studied for the production of lysine. From different concentration of ammonium sulphate, 0.25% was significant ($P < 0.05$) as it produced 16.89 g/L lysine than any other concentration (Fig. 3a). The results of the present study are in line with Adnan [19], who optimized 0.65g ammonium sulphate, 13.0g soybean meal with 0.4% inoculum size to get maximum production of Lysine (23.57g/Kg) through solid state fermentation. Further increase of its concentration showed the decrease of Lysine titre, same findings were observed in the present finding, but there was an increase of biomass concentration. Further it was reported in their findings that combination of soybean meal and $(\text{NH}_4)_2\text{SO}_4$ gave the higher yield of lysine. The results of Nasab [20] and Ekwealor and Obeta [21] also supported that $(\text{NH}_4)_2\text{SO}_4$ as best inorganic nitrogen source but the optimum level they used was 4% of $(\text{NH}_4)_2\text{SO}_4$ that differ from present optimized concentration and also from the findings of Adnan [19]. This difference may be the use of combination of different sources of nitrogen, as urea, $(\text{NH}_4)_2\text{SO}_4$ and corn steep liquor in present study. Among the different concentrations of urea, 0.5% gave the significantly ($P < 0.05$) higher lysine contents (Fig. 3). Mumtaz [22], also used *Brevibacterium flavum* for the fermentation of distillery sludge, with 1% urea, 6% molasses and produced biomass had 24.06% true protein. The concentrations of urea 5g/L was reported as optimum level by Nasab [20] that also supported the optimized concentration of urea in present study. Statistical analysis showed that concentration of NH_4NO_3 , as a nitrogen source has no marked effect to raise lysine production. Furthermore $(\text{NH}_4)_2\text{SO}_4$ having two NH_4^{+1} as compared to NH_4NO_3 that make it more efficient nitrogen source [4]. Corn steep liquor is an agricultural by-product of maize gluten which is a cheap and organic nitrogen source that rich in amino acids, oligopeptides, vitamins and nucleotides [23]. With 0.6 % inclusion of corn steep liquor, lysine concentration was reached to its maximum 19.4 g/L, which was statistically proved as significant ($P < 0.05$) shown in Fig. 3b. The results of Naz [12] are also in line with the present results. They used 4% corn steep liquor and 4% molasses. The amino acid profile revealed 21.48 mg of lysine/g of molasses. The lysine contents was raised to 189 mg/100mL reported by Mumtaz [22] with distillery sludge as substrate, molasses (6%), corn steep liquor (3%) and urea (1%).



Source for hyper- production of Lysine through fermentation with 4% molasses by *Brevibacterium flavum*. (a) NH_4NO_3 (b) Urea (c) $(\text{NH}_4)_2\text{SO}_4$. N stands for nitrogen. Error bars represent increasing percentage of lysine with different nitrogen sources

Fig. 3(a): Optimization of organic and inorganic nitrogen



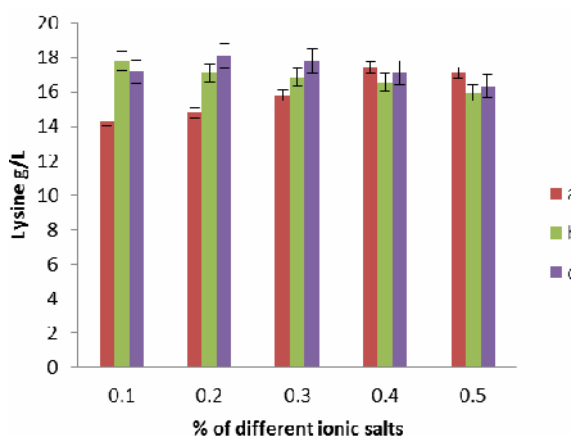
CSL- Corn steep liquor. Error bars represent increasing percentage of Lysine with inclusion of CSL

Fig. 3(b): Optimization of different percentages of CSL for Lysine hyper-production through fermentation with 4% molasses by *Brevibacterium flavum*.

Various concentrations of salts were optimized for maximum lysine production at pH 7 and 30°C. The trend in the (Fig. 4a) gave the optimized concentrations; 0.4% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was found optimum level with lysine titre 17.4g/L and with 0.1% optimum level of NaCl maximum lysine (17.8g/L) was produced. An increased yield of Lysine (18.08 g/L) was observed with 0.2% $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$. The concentrations of above inorganic salts were found to be statistically significant ($P < 0.05$). The results obtained in this study with macro

and micro nutrients are supported by Athar [24]. They used the strain *Candidas utilis* for the biomass production and 0.75% CaCl_2 , 0.125 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.45% $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ was found optimum for lysine production. Sen and Chatterjee [25] reported the effect of different salts NaCl, KCl and MnCl_2 on lysine production and observed maximum lysine concentration with 0.1% NaCl that also supported the present observation. The increased lysine concentration was found with optimization of ionic salts and vitamins. Ekwealor and Obeta [21] described that lysine accumulation was stimulated in the presence of these monovalent, bivalent metal ions and vitamins. Some ingredients of medium, when they are sterilized combine with ionic salts they form complexes that remain insoluble in the medium. Therefore these important constituents remained unavailable for microbial fermentation [23], that's why calcium carbonate (CaCO_3) was dry sterilized and biotin was syringe filtered ($0.22\mu\text{m}$) [10] during their use. From the various concentrations of CaCO_3 , 4% gave significantly high titre (19.01 g/L) of Lysine (Fig. 4b). The medium composition used by Nasab [20] contained 20 g/L CaCO_3 , with different carbon sources for fermentative production of Lysine. Different concentrations of CaCO_3 (20- 50 g) were used by various biotechnologists during fermentation. Liu [26] investigated 30 g/L of CaCO_3 as optimum level in their study. Similarly Shah [27] used different fermentation media having 2% CaCO_3 with maximum Lysine (38, 33 and 28.5g/L) by mutated auxotroph of *Corynebacterium glutamicum*. The results of Shiiro [28] were also coincided with present optimized concentration of CaCO_3 as 50g/L CaCO_3 was reported with 33-36g/L lysine by mutated homoserine and S-(2-aminoethyl)-L-cysteine plus threonine auxotroph of *Brevibacterium flavum*. Furthermore, CaCO_3 has a buffering action in the medium [4] and Ca^{+2} have an important role in the stability of cell wall, cellular enzymes activation and also regulated the cell functions [21]. The production of either glutamic acid or lysine, it was profoundly affected by the concentration of biotin [23]. From the different concentrations of biotin (0.1, 0.2, 0.3, 0.4, 0.5) mg/100mL were optimized for maximum lysine production. With 0.4 mg% of biotin significantly ($P < 0.05$) high yield 19.45 g/L of lysine was obtained. The present study was supported by Ekwealor and Obeta [21], who discussed the importance of biotin for lysine production, and observed the maximum lysine production by *Bacillus megaterium* with 1 $\mu\text{g}/100\text{mL}$ biotin. Tosaka [29] noted the maximum lysine production by increasing biotin concentration from 5 μg - 50 $\mu\text{g}/100\text{mL}$ by *Brevibacterium lactofermentum*. Shiiro [28] supported that lysine accumulation was increased with different

concentrations of biotin by *Brevibacterium flavum* as they used 30 μg and 300 $\mu\text{g}/\text{L}$ biotin with the Lysine production 32g/L and 31g/L respectively. For Lysine accumulation, adequate percentage of biotin is mandatory described by Young and Chipley [30]. It was reported that in the presence of biotin cell up take more glucose than in its absence. The mechanism of glucose uptake was stimulated in the presence of biotin that modified the cell wall to create a charge barrier for excretion of more Lysine extracellular. Sen and Chatterjee [25] also examined the role of biotin in lysine production by *Brevibacterium lactofermentum* ATCC 21086 and *Micrococcus varians*_2fa, respectively and discussed the same findings.



Hyper- production of Lysine through fermentation with 4% molasses by *Brevibacterium flavum*.

(a) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (b) NaCl (c) $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$. Error bars represent increasing percentage of Lysine during optimization of different ionic salts concentrations

Fig. 4: (a)Optimization of different percentages of ionic salts for Hyper- production of Lysine through fermentation with 4% molasses by *Brevibacterium flavum*.

Identification of amino acids is extremely important for amino acid fermentation as well as for evaluation of protein structure. Thin layer chromatography (TLC) is an easy and frequently used technique for the detection and quantification of Lysine. The appearance of violet colour spot having relative flow (R_f) value similar to that of standard lysine confirmed the presence of lysine in the broth (Fig. 5). It showed the presence of Lysine in all the samples of 120h of incubation, but the titre of lysine after 72h of incubation was not increased as quantified by spectrophotometer. Different solvent compositions were used for the detection of lysine [31]. Different Spectrophotometric methods were used for the quantification of Lysine from broth by

different researchers, as in present study quantitative estimation of lysine during the optimization was made accurately by following the method of Chaves [32]. By this method metabolites and vitamins produced during fermentation would not affect the complex formation of Lysine with sodium nitroprusside. Lysine was estimated (17- 18 mg/ 1mL) by Rehman [25] who followed a spectrophotometric method of Hsieh [33] after fermentation by UV- mutated *Corynebacterium glutamicum*. Rao [24] followed the method of Chinard [34] for quantitative assay of Lysine (45.34 g/L) whereas Nasab [20] estimated Lysine 48g/L after 96h of incubation by TLC method.

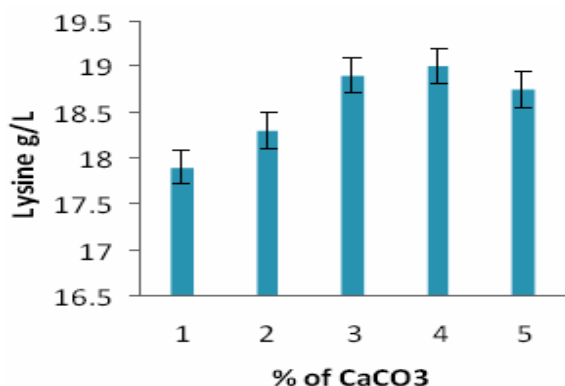


Fig. 4(b): Optimization of different percentages of CaCO₃ for hyper- production of Lysine through fermentation with 4% molasses by *Brevibacterium flavum*.

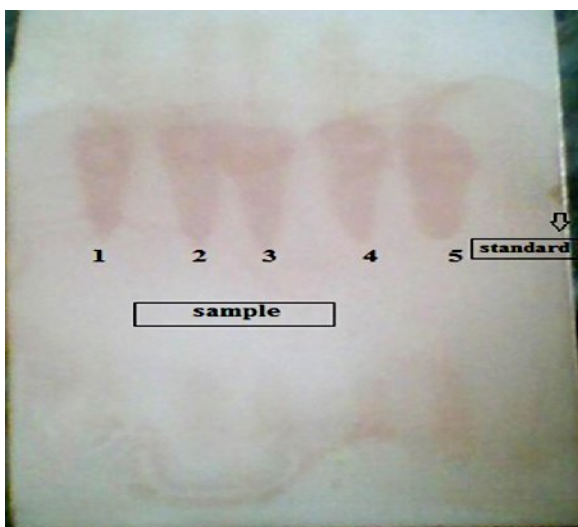


Fig. 5: TLC for the detection of Lysine in fermented broth Samples of different time of incubation showed presence of Lysine that had same R_f value as that of standard. Spot of sample (1) 24 h (2) 48 h (3) 72 h (4) 96 h (5) 120 h. TLC- thin layer chromatography

Conclusion

Agricultural by-products like molasses, wheat bran and rice polishing can be used for production of Lysine and molasses was found to be the best carbon source for Lysine production. In this research a simple and cheap method for lysine production was developed which can be exploited on commercial scale.

Acknowledgment

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