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The Influence of Amino Acids and Trace Elements on L-lysine Production by *Bacillus subtilis* Using Agricultural Products as Carbon and Nitrogen Sources

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

The influence of amino acids and trace elements on L-lysine production by *Bacillus subtilis* using agricultural products as carbon and nitrogen sources was studied. The L-lysine producing bacteria had already been isolated from Nigerian soil. They were purified and identified as *Bacillus subtilis PR13 and B. subtilis PR9*, using cultural, biochemical and molecular characteristics. Optimization of some parameters which included amino acids and trace elements on L-lysine production by *Bacillus* species was carried out. The L-lysine was produced in 100 ml flasks containing fermentation media (FM1 and FM2). The findings revealed that, enhanced lysine yield of

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2.88mg/ml and 1.68 mg/ml by *Bacillus subtilis* PR13 and *Bacillus subtilis* PR9, was observed at in the presence of 0.1% (w/v) of glycine and leucine respectively. There was a negative correlation between amino acid and lysine production by the *B. subtilis* PR 13 (r= -0.74) and PR 9 (-0.55). The supplementation of 0.005 g/l of $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ enhanced optimum L-lysine yield of 2.56 mg/ml for *B. subtilis* PR 13 and 1.36 mg/ml for *B. subtilis* PR9. There was a positive correlation between $MgSO_4.7H_2O$ and lysine production by the *B. subtilis* PR 13 (r=0.96) and $FeSO_4.7H_2O$ and lysine production by *B. subtilis* PR9 (r= 0.94). The results obtained in the study, illustrated that the optimization of process parameters could increase L-lysine yield from agricultural products by *B. subtilis* PR13 and *B. subtilis* PR9.

Keywords: Bacillus species; L-lysine; submerged fermentation; amino acids; trace elements.

1. INTRODUCTION

"L-Lysine is one of the 9 amino acids which is essential for human and animal nutrition. It may be added to food and feed materials to improve the protein quality" [1]. "L-lysine cannot be synthesized biologically in the body and its breakdown is irreversible. In 2015, the world market for L-Lysine was around 2.2 million tons per year" [2]. "It is used as food supplement for humans (children have a high requirement of lysine, since it is needed for bone formation)" [3,4]. "It is utilized in human medicine, in cosmetics and in the pharmaceutical industry, particularly in the formulation of diets with balanced amino acid concentration and in amino acid infusions" [3] and "as precursor for the synthesis of peptides or agrochemicals. Both chemical and biochemical methods are used for L-lysine production" [5,6]. "From the commercially manufactured L-lysine, 80% is manufactured by biochemical method and only 20% by chemical means" [7]. "Among biochemical methods, fermentation is the most economical and practicable means of producing lysine" [8]. "The final product is usually presented as a salt, Lysine-HCl (Lysine monochloridrate)" [5].

L-Lysine is being produced on industrial scale using *Corynebacterium glutamicum*, species of *Arthrobacter* and *Brevibacterium* as fermenting agent [9,10]. High yielding strains have also been developed from *Bacillus subtilis* and *Escherichia coli* [11].

"Agro-industrial by–products are being used as nitrogen and carbon source in lysine production" [12]. "It has been reported that Nigeria has a large production of cassava, cocoyam, millet, potato, plantain, yam, rice, corn, wheat, sorghum, soy bean, pigeon pea, cowpea, Bambara, sugar cane and groundnut" [13]. Some of the carbon sources (which contain sucrose, glucose and fructose) provide a source of fermentable sugars as well as some elemental nutrients, which plays key role in the fermentation process.

As Nigeria is a developing country, a huge amount of foreign exchange is spent in the importation of L-lysine for its local industries. There is huge potential in production of L-lysine locally by microbiological methods using available agricultural products. Because of the availability of these agricultural products in Nigeria, lysine production by fermentation process may likely be more economical.

"Extensive research has been made in order to improve the fermentation process not only from the point of lowering production costs but also of increasing productivity" [14]. "Improvements have included for example, increased yield of desired metabolites, removal of unwanted co-metabolites, improved utilization of inexpensive carbon and nitrogen sources, or alteration of the morphology to a form better suited for separation of the organisms from the product" [14].

"In an earlier study, we had isolated three *Bacillus* species (which included *Bacillus subtilis* PR13, *Bacillus subtilis* PR9, and *Bacillus pumilus* SS16) from Nigerian soil, which produced various yields of L-lysine" [15]. "In another study, the *Bacillus* species were used for L-lysine production using carbohydrates as carbon and seed meals as nitrogen sources" [16].

The present research work was aimed at determining the influence of amino acids and trace elements on L-lysine production by *Bacillus subtilis* using agricultural products as carbon and nitrogen sources.

2. MATERIALS AND METHODS

2.1 Isolation of Bacteria

The two bacterial isolates used in this study were isolated from different locations in Awka town,

Anambra state, Nigeria [15]. They were purified and Identified as *B. subtilis* PR13 and *B. subtilis* PR9, using cultural, biochemical and molecular characteristics. The bacteria cultures were maintained at 4°C until used and examined for L-lysine production.

2.2 Inoculum Preparation

Two loopful of *B. subtilis* PR13 and PR9 were inoculated in an Erlenmeyer flask containing 50 ml of seed medium which had already been sterilized at 121^{o}C for 15 min. The seed medium consisted of peptone, 10.0g; yeast extract, 10.0 g; NaCl, 5.0 g; water, 1litre; pH was adjusted to 7.2. The inoculated flasks were incubated for 24 h on a rotary shaker at 120 rpm and 30^{o}C. Duplicate flasks were used.

2.3 Media for Fermentation

Two different fermentation media (FM1 and FM2) were used by the two *Bacillus* species for L-lysine production. A cotton plugged Erlenmeyer flasks (100ml) containing 20ml of fermentation medium (FM1) comprising of KH_2PO_4, 0.5g; K_2HPO_4, 0.5g; $MgSO_4.7H_2O$, 0.001g; $MnSO_4.H_2O$, 0.001g; $FeSO_4.7HO$, 0.001g; $CaCO_3$, 50g, the carbon source (glucose) was replaced with millet starch hydrolysate 60g; the nitrogen source (ammonium sulphate) was replaced by soyabean meal 40g; water, 1 litre; pH was adjusted to 7.2, was used for *Bacillus subtilis* PR13. Another cotton plugged Erlenmeyer flasks (100) containing 20ml of fermentation medium (FM2) comprising of KH_2PO_4, 0.5g; K_2HPO_4, 0.5g; $MgSO_4.7H_2O$, 0.001g; $MnSO_4.H_2O$, 0.001g; $FeSO_4.7HO$, 0.001g; $CaCO_3$, 50g, the carbon source (glucose) was replaced with sorghum hydrolysates 60g, the nitrogen source (ammonium sulphate) was replaced by defatted peanut meal (an agricultural product) 40g; water, 1 litre; pH was adjusted to 7.2, was used for *B. subtilis* PR9.

2.4 Optimization of Culture Conditions for L-lysine Production

2.4.1 Effect of amino acids

The effect of amino acids on growth and L-lysine production were determined. A (100ml) Erlenmeyer flasks containing the 20 ml of fermentation media (FM1 and FM2) as was previously described, was used for L-lysine production. Various amino acids, which included 0.01% (w/v) of glycine, tryptophan, methionine, leucine, aspartic acid, threonine and isoleucine were added to the fermentation media and sterilized at 121^{o}C for 15 min. After sterilization, the media were cooled to room temperature and 1ml (1.8×107 cfu/ml) volume of the cultures of *Bacillus* species (24 h) was inoculated into the fermentation media. Uninoculated flasks served as control. The flasks were placed on a rotary shaker (at 160 rpm) and incubated at 30^{o}C for 72 h. At the end of incubation, samples of the fermentation medium were aseptically dispensed into cuvettes using micropipettes. Thereafter, the cuvettes were placed in the spectrophotometer and the reading for bacteria growth was determined at 660 nm. For the determination of L-lysine and residual sugar, the fermentation medium was subjected to centrifugation at 5,000 rpm for 15 min to obtain the cell free supernatant which is the crude L-lysine. The cell free supernatant was used for the determination of lysine and residual sugar. The experiments were conducted in triplicates.

2.4.2 Effect of trace elements

The effect of trace elements on growth and lysine production by *B. subtilis* PR13 and PR9 was studied. A (100ml) Erlenmeyer flasks containing the 20 ml of fermentation media (FM1 and FM2) as was previously described, was used for L-lysine production. Various concentrations (0.001 – 0.005g/l) of $MgSO_4.7H_2O$, $MnSO_4.7H2O$ and $FeSO_4.7H_2O$ were added to the fermentation media and sterilized at 121^{o}C for 15 min. After sterilization, the media were cooled to room temperature and 1ml (1.8×10^{7}) volume of the cultures of Bacillus species (24h) was inoculated into the fermentation media. . Uninoculated flasks served as control. The flasks were placed on a rotary shaker (at 160 rpm) and incubated at 30^{o}C for 72h. Thereafter, residual sugar, bacterial growth and L-lysine production were determined from the broth culture. The experiments were conducted in triplicates.

2.5 Quantitative Determination of Lysine

L-lysine in the broth culture was determined by acidic ninhydrin method of Chinard [17]. A 5ml volume of the culture broth of the isolate was centrifuged at 5000 ×g for 20min, and the cell-free supernatant was collected and assayed for lysine production.1ml of glacial acetic acid was added to 1ml of supernatant in a test tube. Thereafter, one ml of a reagent solution which contains an acid mixture, 0.4ml of 6M orthophosphoric acid, 0.6ml of glacial acetic acid and 25mg of ninhydrin, was also added to the

supernatant in the test tube. The blank contains 1ml of glacial acetic acid, 1ml of the acid mixture without ninhydrin and 1ml supernatant. Both tubes were capped and the contents mixed properly for 10min before heating at 100^{o}C in a water bath for 1h. The test tubes were cooled rapidly under tap water and 2ml of glacial acetic acid was added to each test tube to give a final volume of 5ml.The optical density of the reacting mixture was read against the blank at 515nm in a spectrophotometer. Results obtained with the test samples were extrapolated from a standard lysine curve.

2.6 Estimation of Reducing Sugar

The reducing sugar content was determined by dinitrosalicyclic acid (DNS) method of Miller [18]. Reducing sugar was estimated by adding 1ml of DNS to 1ml of the supernatant. The mixture was heated in a water bath at 100 ^{o}C for 10min and allowed to cool. The volume of the mixture was thereafter increased to 12 ml with distilled water. After allowing the reaction mixture to stand for 15min at room temperature, the optical density was measured at 540 nm in a spectrophotometer against a blank prepared by substituting the supernatant with water. The reducing sugar content was subsequently determined by making reference to a standard curve of known glucose concentrations.

2.7 Statistical Analysis

Data generated from this work were analyzed using correlation analysis with a software application SPSS version 14.

3. RESULTS

The effect of amino acids on growth and lysine production by *Bacillus subtilis* PR13 and *Bacillus subtilis* PR9 is shown Figs. 1 and 2. The highest lysine production by *Bacillus subtilis* PR13 and *Bacillus subtilis* PR9 was observed at the supplementation of 0.1% w/v of glycine and leucine respectively. The highest lysine accumulation, corresponded with a residual sugar of 0.41 and 0.48 mg/ml respectively. Aspartic acid, methionine, threonine, lysine and isoleucine did not stimulate growth and lysine production in all the *Bacillus* species. There was a negative correlation between amino acid and lysine production by the *B. subtilis* PR 13 (r= -0.74) and PR 9 (-0.55).

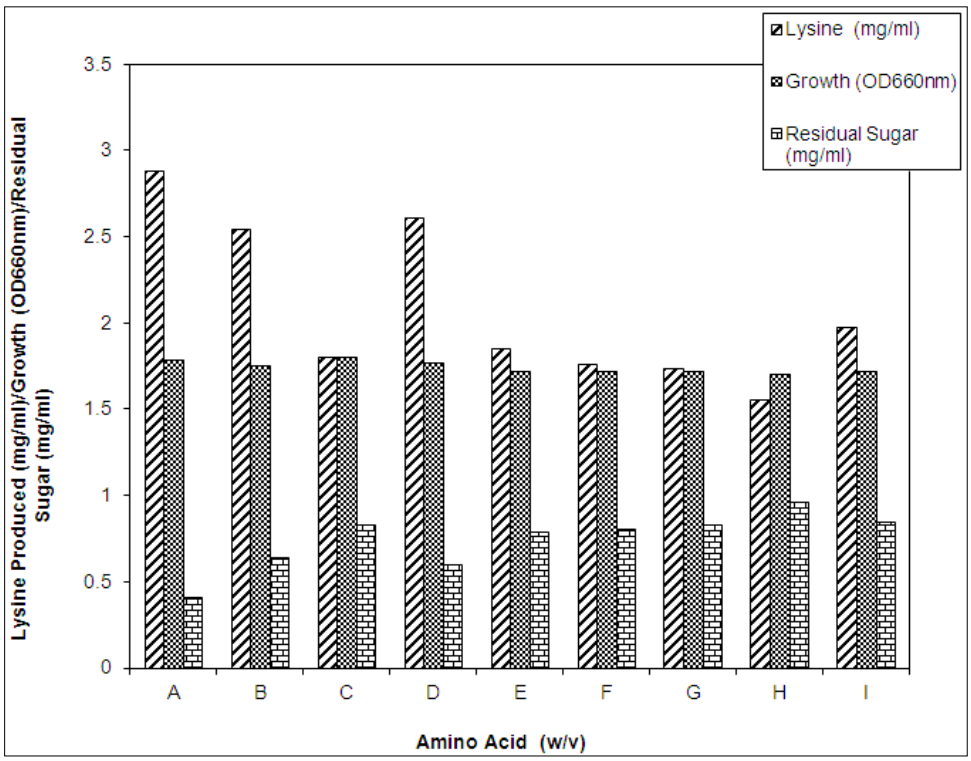

Fig. 1. Effect of Amino Acids on Lysine Production by *Bacillus subtilis* PR13: A, Glycine; B, Trytophan; C, Methionine; D, Leucine; E, Aspartic Acid; F, Threonine; G, Isoleucine; H, Lysine; I, Control

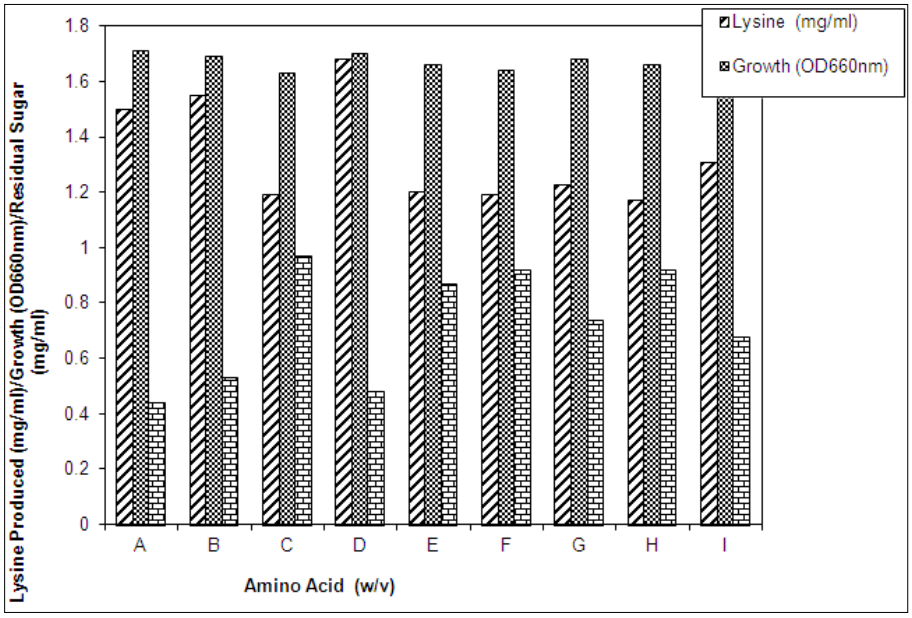

Fig. 2. Effect of Amino Acids on Lysine Production by *Bacillus subtilis* PR9:A, Glycine; B, Trytophan; C, Methionine; D, Leucine; E, Aspartic Acid; F, Threonine; G, Isoleucine; H, Lysine; I, Control

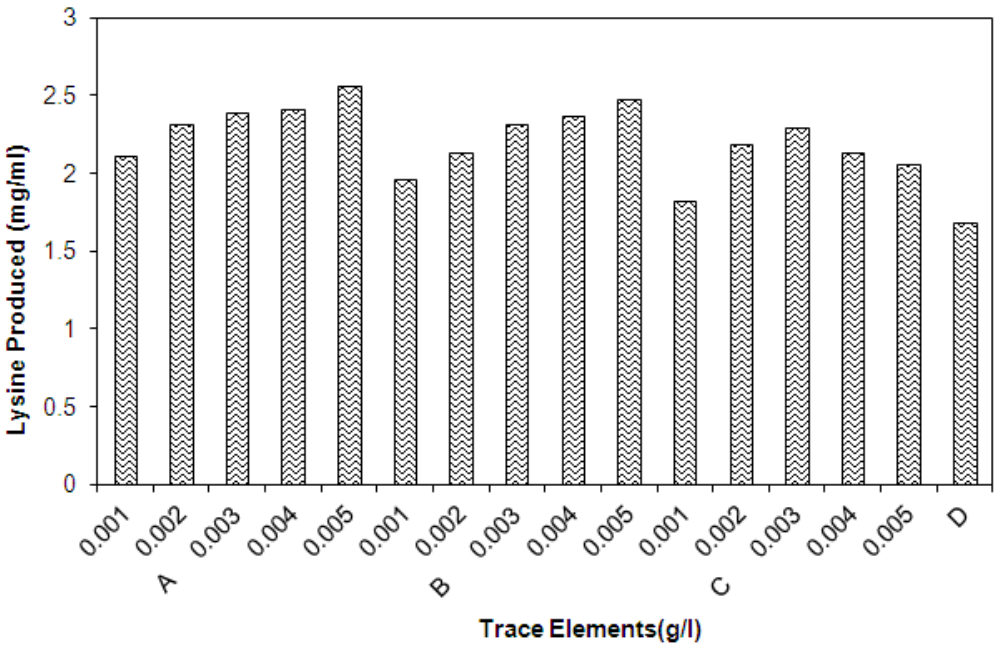

Fig. 3. Effect of Trace Elements on Lysine Production by *Bacillus subtilis* PR13 A, MgSO4.7H2O; B, MnSO4.7H2O, C, FeSO4.7H2O; D, Control (without trace elements)

The effect of trace elements on growth and lysine production by *B. subtilis* PR13 and PR 9 are presented in Figs. 3-4.The results showed that maximum lysine yields by *Bacillus subtilis* PR13 and B. subtilis PR9, were observed at the addition of 0.005 g/l of $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ respectively. The highest lysine accumulation of 2.56 and 1.36 mg/ml was observed for *B. subtilis* PR13 and PR9 respectively. There was a positive correlation between $MgSO_4$.7H2O and lysine production by the *B. subtilis* PR 13 (r=0.96), while there was a positive correlation between $FeSO_4.7H_2O$ and lysine production by *B. subtilis* PR9 (r= 0.94).

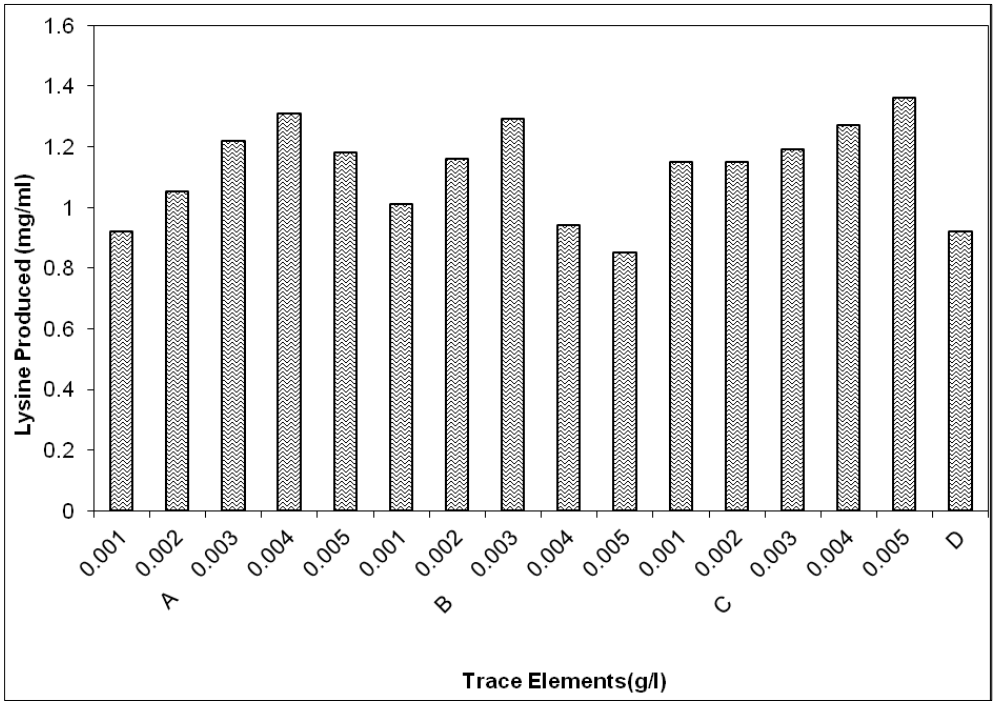

Fig. 4. Effect of Trace Elements on Lysine Production by *Bacillus subtillis* PR9: A, $MgSO_4$.7H2O; B, MnSO4.7H2O, C, FeSO4.7H2O; D, Control(without trace elements)

4. DISCUSSION

The results from the study showed that glycine and leucine stimulated maximum lysine yield in *Bacillus subtilis* PR13 and PR9 respectively. This is contrary with the work of Ezemba et al. [19], who noted that L- methionine enhanced L-lysine production in *Microbacterium lacticum.* Shio and Uchio [20], also observed that L-methionine enhanced the L-glutamic acid production by *Corynebacterium hydrocarboclastus* R-7. It was observed in the study, that members of the aspartate family, which included aspartic acid, methionine, threonine and isoleucine, did not stimulate growth and enhanced lysine accumulation. The inhibitory effect of the aspartate family, as suggested by Mindlin and Zaitseva [21], may be due to repression or inhibition of specific enzymes which direct the biosynthetic pathway for producing only lysine. The fact that the addition of lysine also inhibited formation of lysine extensively indicates the presence of Feedback regulation in the pathway. Yamada et al. [22] reported that the addition of methionine inhibited the formation of methionine by Methylotroph strain OM-33. Zaitseva and Konovalova [23] studied "the effect of threonine on growth and lysine production using four homoserine dependent mutants viz; *Corynebacterium glutamicum* 95 and *Brevibacterium* spp-22 (sensitive) and *Corynebacterium glutamicum* 1020-60 and 410-6 (resistant). The L-lysine accumulation was proportional to the threonine content".

Results from the study revealed that $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ encouraged optimum production of lysine. Shah et al. [3] tested the effects of different amounts of magnesium sulfate, ferrous sulfate and manganese chloride on lysine production by *Corneybacterium glutamicum*. They observed that maximum yield was obtained at 50mg per 100ml for magnesium sulfate (16g/l L-lysine), and 0.2mg per 100ml each of $FeSO_4$ and $MnCl_2$. Sen and Chatterjee [9] further studied "the effect of trace elements on L-lysine production by Micrococcus varians 2fa, which produced 2.6g/l L-lysine. Addition of trace elements to the optimal media has been found to stimulate growth and enhance L-lysine yield". Rao et al. [24], Umerie et al. [25] and Ekwealor and Obeta [8], used different concentration of trace elements in their fermentation media for amino acid production. Trace elements facilitate the transport of materials across cell membrane. Fe^{2+} and Mn^{2+} are the most important of the trace elements as they play a role in the excretion of primary metabolites. Metal ions play a vital role in fermentation as they are co-factors for various

enzymes. They are required to activate enzymes [26].

5. CONCLUSION

The study showed that some agricultural products could be harnessed as good substrates for L-lysine production by submerged fermentation. During the optimization study, which included influence of addition of amino acids and trace elements, it was observed that there was improved L-lysine production by *B. subtilis* PR13 and *B. subtilis* PR9. The concentrations of 0.1% w/v of glycine and leucine and 0.005 g/l of $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ were optimal for L-lysine production by *B. subtilis* PR13 and PR9 respectively. However, *B. subtilis* PR13 produced the maximum concentration of L-lysine yield. The *Bacillus* species have shown potential for L-lysine production using readily available agricultural products. These products are good sources of carbon and nitrogen and are rich in fermentable substrates. This development indicates that large scale L-lysine production is feasible in Nigeria and it will help to meet present-day needs in the Nigeria's industrial sector.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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