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The Influence of Vitamins, Antibiotics and Growth Stimulators 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

L-Lysine is one of the 9 amino acids which are essential for human and animal nutrition. It is a basic building block of all proteins and cannot be synthesized biologically in the body. The influence of vitamins, antibiotics and growth stimulators on L-lysine production by *Bacillus subtilis* using agricultural products as carbon and nitrogen sources was examined. The L-lysine producing bacteria had already been isolated from Nigerian soil. They were purified and Identified as *B. subtilis* PR13 and *B. subtilis* PR9, using cultural and biochemical characteristics. The of influence of vitamins, antibiotics and growth stimulators on L-lysine production by *Bacillus subtilis* PR13 and

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9 was evaluated in 100 ml flasks containing 20 ml fermentation media (FM1 and FM2). The results obtained showed that, an enhanced lysine yield of 3.41 and 1.57 mg/ml by *Bacillus subtilis* PR13 and *Bacillus subtilis* PR9, was observed in the presence of 1 µg/ml of biotin and 10 µg/ml respectively. The supplementation of 0.04 units/ml of penicillin, enhanced optimum L-lysine yield of 2.38 mg/ml for *B. subtilis* PR 13 and 1.64 mg/ml for *B. subtilis* PR9 respectively. The addition of 0.1% w/v peptone and yeast extract, enhanced optimum L-lysine yield of 2.66 mg/ml for *B. subtilis* PR 13 and 1.72 mg/ml for *B. subtilis* PR9 respectively. There was a positive correlation between peptone and L-lysine production by *B. subtilis* PR13 (r= 0.85) and yeast extract and L- lysine production by *B. subtilis* PR9 (r= 0.54). The results obtained in the study showed that the supplementation of the fermentation media with some vitamins, antibiotics and growth stimulators enhanced the L-lysine yield of *B. subtilis* PR13 and *B. subtilis* PR9.

Keywords: Bacillus subtilis; L-lysine; submerged fermentation; vitamins; antibiotics.

1. INTRODUCTION

"Amino acids are major industrial products derived by fermentation, covering a world market of more than 5 million tons per year, and among amino acids is the L-lysine that is one of the leading biotechnological products with a current production of 2.2 tons per year" [1].

"L-Lysine is an amino acid primarily essential for human and animal nutrition, and is usually obtained in batch or fed-batch fermentation processes. Of that manufactured commercially, the largest amount of 80% is produced by fermentation and 20% by chemical synthesis" [2]. "Lysine is used as food (flavour) enhancer and also food preservation especially with ε-poly-L-lysine" [3]. "It is used as food supplements for humans (children have a high requirement of lysine, since it is needed for bone formation)" [4, 5]. "It is used to enrich feed stuff in order to provide an adequate diet for poultry, cattle and other livestock. Animal feed such as grain and defatted oil seeds, contain only a small quantity of lysine" [6].

"The use of L- lysine is helpful to overcome angina pectoris. It is an essential ingredient used to clean arteries, important for cancer prevention" [7]. "Lysine supports bone health by ensuring adequate absorption of calcium and therefore prevents osteoporosis. It has an important role in production of antibodies for healthy immune system. It is the integral component of musculature" [8]. "In addition, it has pharmaceutical applications both in the formulation of diets with a balanced amino acid composition and in the infusion of amino acids" [9] A great variety of microorganisms have been discovered to produce L-lysine on industrial scale. They include *Corynebacterium glutamicum*, *Bacillus subtilis, Bacillus megaterium*, *Escherichia coli,* species of *Arthrobacter, Brevibacterium and Micrococcus* [10-13].

"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. 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]. "Utilization of agricultural by-products as substrates for fermentation might offer an inexpensive alternative for microbial products such as amino acids" [15,16,17].

Currently, Nigeria meets all its L-lysine needs only through importation. However, L-lysine can be made available and more economical if produced locally by fermentation using agricultural products. A huge amount of foreign exchange will be saved and this development will impact positively on the economy.

We had isolated three *Bacillus* species (which included *Bacillus subtilis* PR13, *B. subtilis* PR9 and *B. pumilus* SS16) from Nigerian soil and they produced various yields of L-lysine [18]. In another study, the *Bacillus* species were used for L-lysine production using carbohydrates as carbon and seed meals as nitrogen sources [19].

2. MATERIALS AND METHODS

2.1 Microorganisms and Culture Maintenance Conditions

The *Bacillus subtilis* PR13 and *B. subtilis* PR9 that were isolated from different soil in Awka

town, were used in the study. They were purified and Identified as *B. subtilis* PR13 and *B. subtilis* PR9, using cultural and biochemical characteristics. The *Bacillus subtilis* were grown on nutrient agar slants for 24 h at 37^{o}C. Thereafter, the cultures were then preserved at 4^{o}C and transferred to new slants after 30 days in order to keep them viable for use in L-lysine production.

2.2 Seed Culture Preparation

The seed medium consisted of peptone, 10.0g; yeast extract, 10.0 g; NaCl, 5.0 g; water, 1litre; pH adjusted to 7.2. Two loopfuls 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 inoculated flasks were incubated for 24 h on a rotary shaker at 120 rpm and 30 ^{o}C. Duplicate flasks were used.

2.3 Fermentation Media Preparation

The submerged production of L-lysine by *Bacillus subtilis* PR13 and PR9, was conducted in two fermentation media namely fermentation medium 1 and 2 (FM1 and FM2). For *Bacillus subtilis* PR 13, L-lysine production was carried out in 100 ml Erlenmeyer flasks, containing 20ml of fermentation medium 1 (FM1). The medium, was composed 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 adjusted to 7.2. For *Bacillus subtilis* PR 9, fermentation medium (FM2) was used for L-lysine production. FM2 is similar to FM1, except that the carbon source (glucose) was replaced with sorghum hydrolysates 60g, the nitrogen source (ammonium sulphate) was replaced by defatted peanut meal 40g. The carbon source substrates were prepared in the laboratory using the method of Umerie et al. [20].

2.4 Optimization of Culture Conditions for L-lysine Production

2.4.1 Effect of vitamins

The effect of vitamins, which included various concentrations (0.01-100µg/ml) of riboflavin, thiamine, biotin, nicotinic acid and cyanocobalamin on lysine production was determined. Fermentation was carried out in 100ml Erlenmeyer flasks containing 20 ml of fermentation media (FM1 and FM2) as was previously described. The vitamins were added to the fermentation media and sterilized at 121^{o}C for 15 min. One milliliter volume (1.8×10^{7} cfu/ml) of 24h cultures of *Bacillus* species 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. Following the termination of fermentation, the culture broth 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. The experiments were conducted in triplicate.

2.4.2 Effect of antibiotics

The effect of antibiotics, which included varying concentrations (0.01 – 0.10units/ml) of penicillin and (0.01 – 2.00µg/ml) of vancomycin, erythromycin, lincomycin, tetracycline and chloramphenicol on lysine production was studied. Fermentation was carried out in 100ml Erlenmeyer flasks containing 20 ml of fermentation media (FM1 and FM2) as was previously described. The antibiotics were added to the fermentation media and sterilized at 121^{o}C for 15 min. One milliliter volume (1.8×10^{7} cfu/ml) of 24h cultures of *Bacillus* species 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. Following the termination of fermentation, the culture broth 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. The experiments were conducted in triplicate.

2.4.3 Effect of growth stimulators

The effect of growth stimulators ,which included various growth stimulators which included 0.1% w/v of gelatine, yeast extract, peptone, tryptone, casein and beef extract was carried out. Fermentation was carried out in 100ml Erlenmeyer flasks containing 20 ml of fermentation media (FM1 and FM2), as was previously described. The growth stimulators were added to the fermentation media and sterilized at 121^{o}C for 15 min. One milliliter volume (1.8×10^{7} cfu/ml) of 24h cultures of *Bacillus* species 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°C for 72 h. Following the termination of fermentation, 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 660nm. 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 triplicate.

2.5 Quantitative Determination of Lysine

"L-lysine in the broth culture was determined by acidic ninhydrin method of Chinard" [21]. "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. One milliliter (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" [21].

2.6 Estimation of Reducing Sugar

"The reducing sugar content was determined by dinitrosalicyclic acid (DNS) method of Miller" [22]. "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" [22].

2.7 Statistical Analysis

Data generated from this work were analyzed using correlation analysis with a software application statistical package for social sciences (SPSS) version 14.

3. RESULTS

3.1 Effect of Growth Stimulators

The results of the effect of growth stimulators on growth and lysine production by *Bacillus subtilis* PR13 and *Bacillus subtilis* PR9 are shown Figs. 1 and 2. The highest L- lysine production of 2.66 and 1.72 mg/ml by *Bacillus subtilis* PR13 and *Bacillus subtilis* PR9 were observed at the supplementation of 0.1 % w/v of peptone and yeast extract respectively. The highest yield corresponded with a final reducing sugar of 0.75 for *B. subtilis* PR13 and 0.55 mg/ml for *B. subtilis* PR9. There was a positive correlation between peptone and lysine production by *B. subtilis* PR13 (r= 0.85) and yeast extract and lysine production by *B. subtilis* PR9 (r= 0.54).

3.2 Effect of Vitamins

The results of the effect of vitamins on lysine production by *B. subtilis* PR13 and PR 9 are presented in Figs. 3-4. The results showed that maximum lysine yields of 3.41 and 1.57 mg/ml by *Bacillus subtilis* PR13 and *B. subtilis* PR9, were observed at the addition of 1 and 10 µg/ml of biotin and nicotinic acid respectively. There was a negative correlation between biotin and lysine production by *B. subtilis* PR13 (r= -0.99), while there was a positive correlation between nicotinic acid and lysine production by *B. subtilis* PR9 (r= 0.38).

3.3 Effect of Antibiotics

The results of the effect of antibiotics on lysine production by *B. subtilis* PR13 and PR 9 are presented in Figs. 5-6. The results showed that maximum lysine yields of 2.38 and 1.64 mg/ml by *Bacillus subtilis* PR13 and *B. subtilis* PR9, were observed at the addition of 0.04 units/ml of penicillin. There was a negative correlation between penicillin and lysine production by the *Bacillus subtilis* PR 13 and 9 (r= -0.90, and -0.90) respectively.

4. DISCUSSION

Biotin stimulated lysine yield was found as a requirement for growth and lysine production. Shah et al. [23] studied the effects of biotin on lysine production by *Corneybacterium glutamicum* and observed that maximum lysine was produced at 5µg per 100ml of biotin. A gradual increase in the level of biotin in the medium stimulated growth and methionine production. Tosaka et al. [24] investigated "the role of biotin levels on L-lysine formation in *B.lactofermentum*. They reported that accumulation of L-lysine was stimulated considerably by increasing the biotin level". Zaki et al. [25] worked on "the effect of non-ionic detergents and vitamins on the production of amino acids by *Bacillus ammoniagenes*. They found that the presence of 20µg/l biotin induced the production of about 166mg% L-lysine". Also Banik and Majumdar [26] reported "a maximum methionine production by an auxotrophic mutant of *Micrococcus glutamicus* at a biotin level suboptimal for growth. A further increase in the biotin level stimulated growth, but not methionine production. The exact role of biotin in the amino acid production by microorganisms is not clear". While Tanaka et al. [27] believe that "biotin functions by limiting growth and allowing the carbon and nitrogen sources to the formation of amino acids rather than to the synthesis of cell matter", Shiio et al. [28], Veldkamp et al. [29] and Otsuka et al. [30] believed that "a low biotin concentration makes the bacterial cells more permeable resulting in a higher leaching out of amino acids into the surrounding medium". Beker [31] developed "threonine- methionine double auxotrophic mutant of *Brevibacterium flavum* which required biotin. He found that at low concentration of biotin, biosynthesis of glutamic acid takes place and intensive synthesis of L-lysine can be observed at the beginning of the stationary phase of growth". Young and Chipley [32] investigated "the role of biotin in L-lysine production in *Brevibacteium lactofermentum.* They observed that biotin treated cell took up more glucose, than did the control one. Biotin apparently, caused some compositional changes in the cell wall membrane complex, allowing an increase in uptake of glucose.The result of uptake studies and fatty acid analysis suggested that biotin affected the cell surface, probably the bacterial membrane. It is well known that bacterial membrane plays an important role as a charged barrier. This mechanism might also regulate the amount of L-lysine released by the cells".

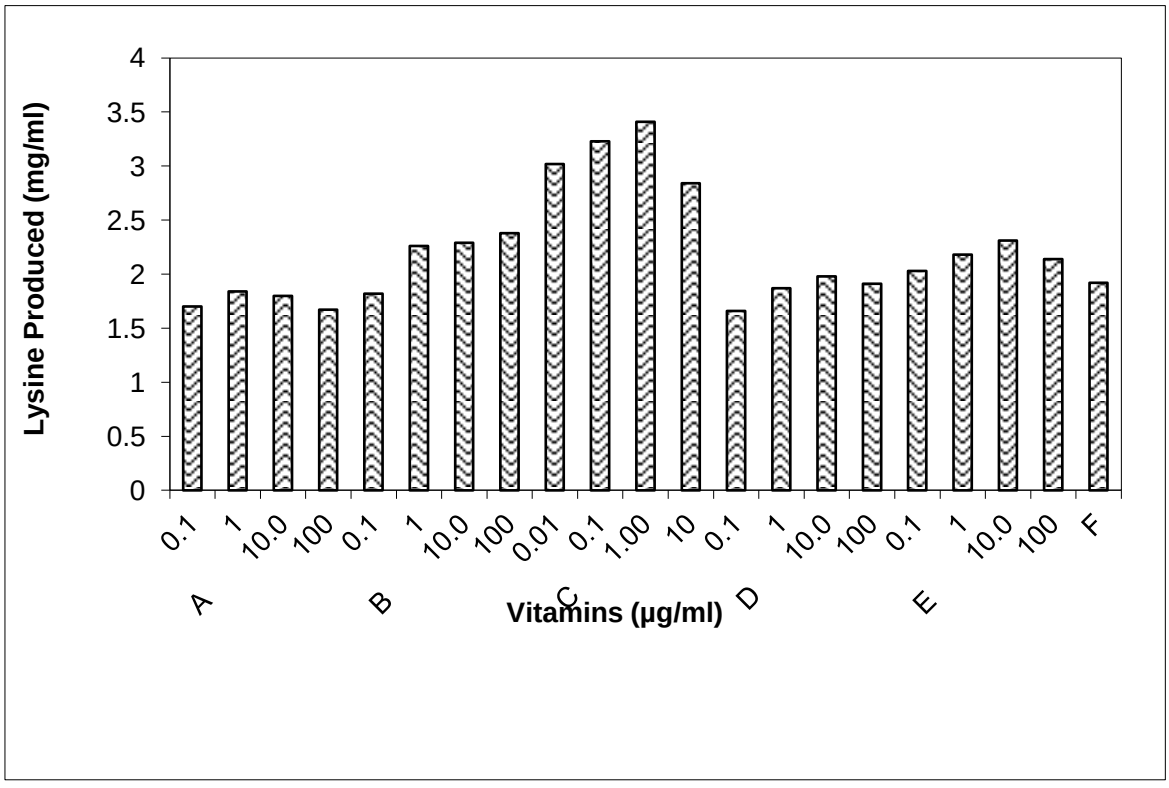

Fig. 1. Effect of vitamins on lysine production by *Bacillus subtilis* PR13: A, riboflavin; B, thiamine; C, Biotin; D, nicotinic Acid; E, cyanocobalamin; F, control(without vitamins)

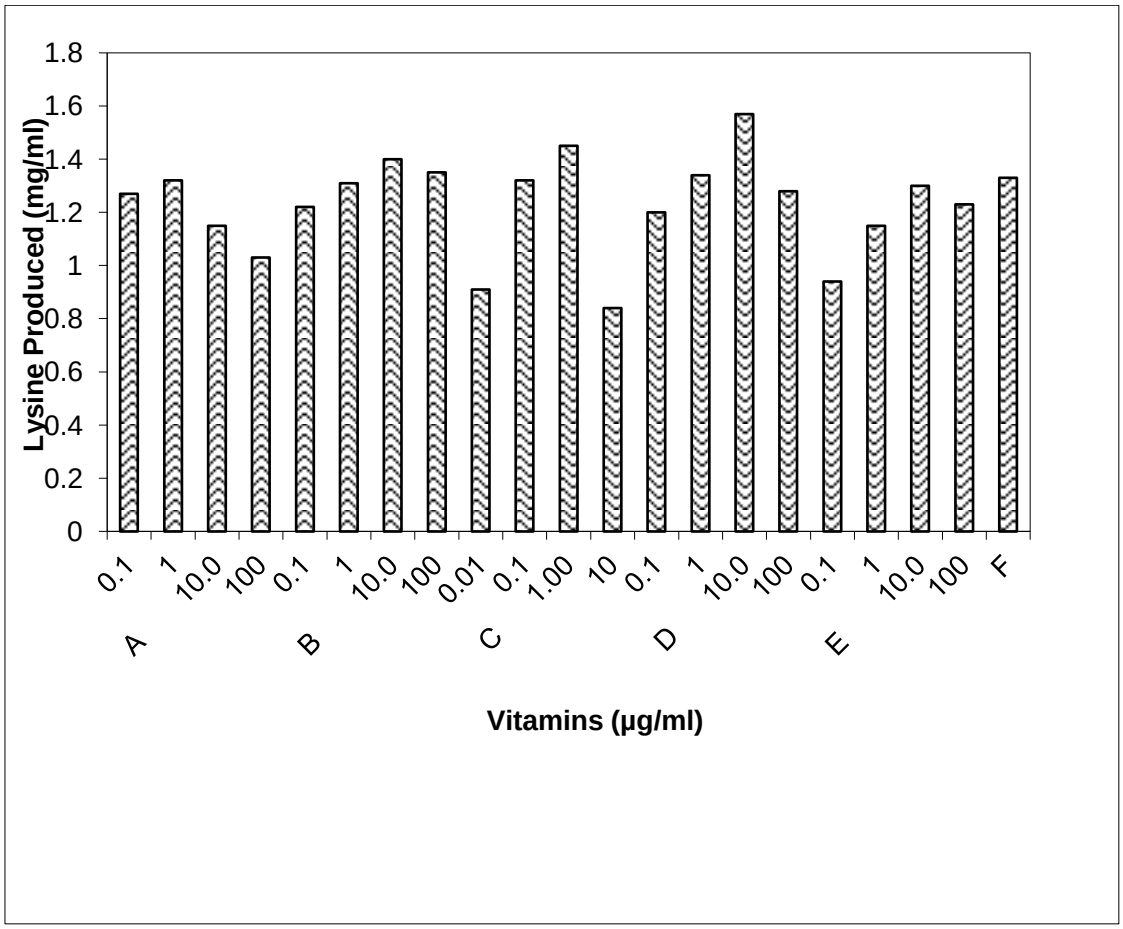

Fig. 2. Effect of vitamins on lysine production by *bacillus subtilis* PR9 A, riboflavin; B, thiamine; C, biotin; D, nicotinic acid; E, cyanocobalamin; F, control(without vitamins)

The antibiotics stimulated different degree of lysine production. Penicillin encouraged lysine production in all the strain. Sen and Chatterjee [33] tested the effects of different antibiotics on lysine production by *Arthrobacter globiformis* and observed that they stimulated growth and lysine production. Vancomycin stimulated lysine production in *Bacillus subtilis* PR9 and *Bacillus pumilus* SS16. This is in agreement with the findings of Ekwealor and Obeta [34] who reported increase in lysine production in *Bacillus megaterium* when vancomycin was used.

Tetracycline stimulated lysine production in *Bacillus subtilis* PR13. Zaki et al. [35] reported that 22-24g/l lysine was produced by *Micrococcus glutamicum* when tetracycline was added to the fermentation medium. Demian and Brinbum [36] suggested "a change in permeability of cell wall caused by antibiotics which may be responsible for improved amino acid yield. This change in permeability affects the intercellular accumulation of amino acids, with the result that the amino acid can no longer regulate its own synthesis by feed back control, thereby releasing high levels of amino acid into the medium. Chloramphenicol did not stimulate lysine production in *Bacillus subtilis* PR9". Israelides et al. [37] reported that "In immobilized cell preparations, growth of cells outside the immobilization matrix, as free cells, is normally undesirable due to the appearance of cells in the product stream and clogging of such systems. Antibiotics could be used to arrest such free cell growth, while allowing the synthesis and excretion of the product into the medium. Chloramphenicol at 200 µg/ml effectively arrests free cell growth and hence the L-lysine being produced can be entirely attributed to the immobilized cells. Novobiocine on the hand at concentration of 100µg/ml, stopped free cell growth, and also prevented the production of L-lysine. Productivity and yields of L-lysine were adversely affected by chloramphenicol and novobiocin probably due to a great decrease in cell viability".

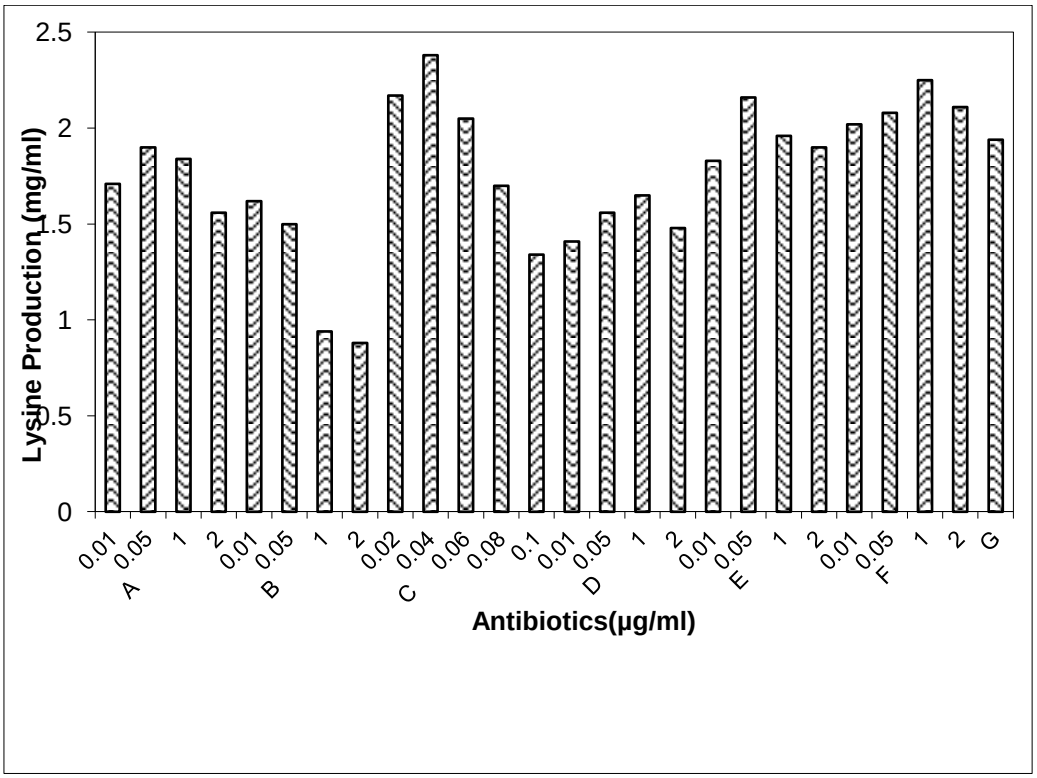

Fig. 3. Effect of antibiotics on lysine production by *bacillus subtilis* PR13: A, vancomycin; B, erythromycin; C, penicillin (units/ml); D, lincomycin; E, tetracycline; F, chloramphenicol; G, control(without antibiotics)

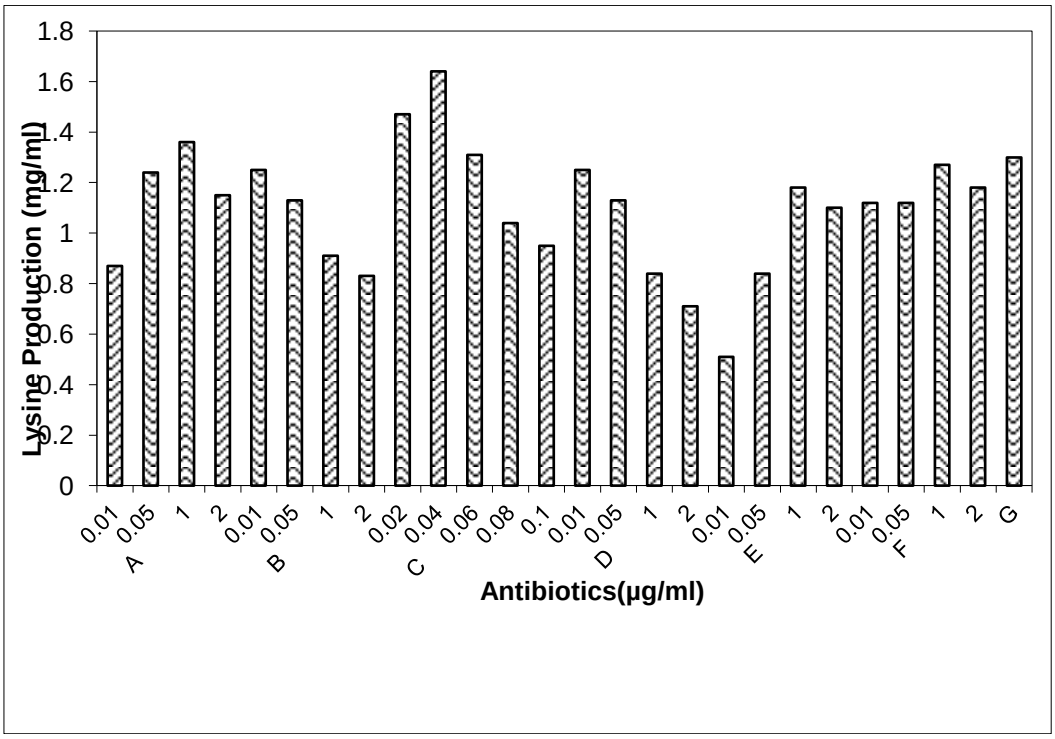

Fig. 4. Effect of antibiotics on lysine production by *bacillus subtilis* PR9: A, vancomycin; B, erythromycin; C, penicillin (units/ml); D, lincomycin; E, tetracycline; F, chloramphenicol; G, control(without antibiotics)

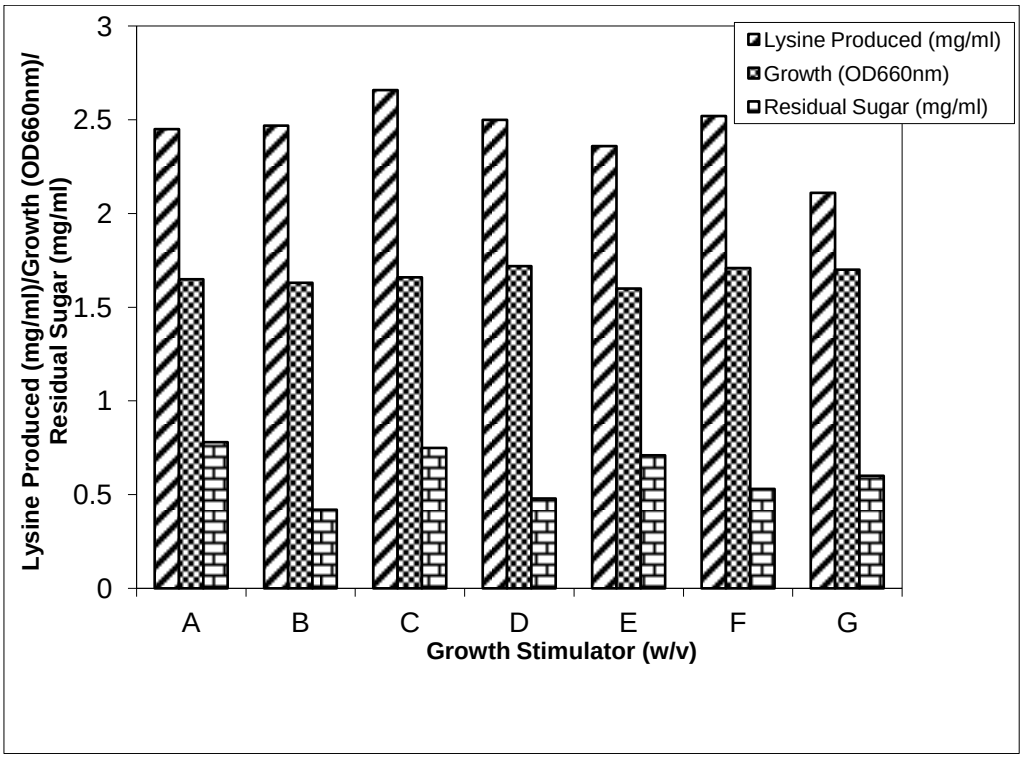

Fig. 5. Effect of growth Stimulators on lysine production by *Bacillus subtilis* PR13: A, gelatine; B, yeast Extract; C, peptone; D, tryptone; E, casein; F, beef extract; G, Control.

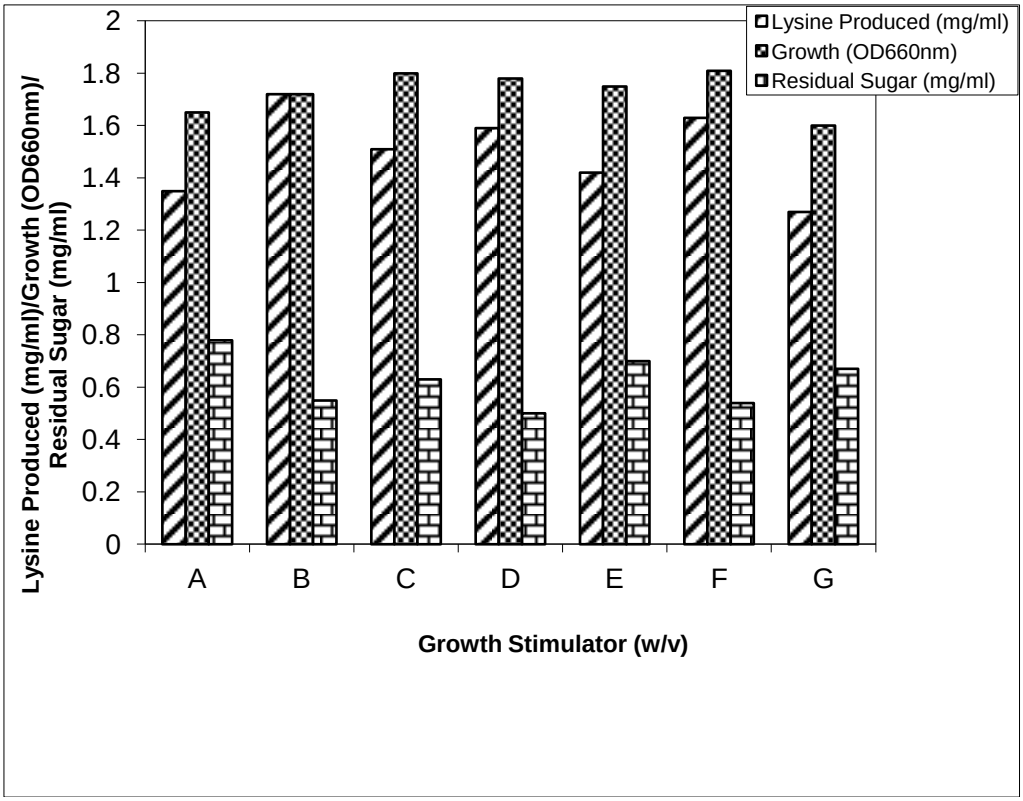

Fig. 6. Effect of growth Stimulators on lysine production by *Bacillus subtilis* PR9: A, Gelatine; B, yeast extract; C, peptone; D, tryptone; E, Casein; F, beef Extract; G, control

It was observed from the study that yeast extract stimulated the highest lysine yield in *Bacillus subtilis* PR9. This is in line with the report of Morinaga et al. [38], who observed an increase in lysine production by *Pseudomonas* species 518 when 7.5mg/ml of yeast extract was added. Ekwealor and Obeta [39] reported that the growth promoting substances used retarded growth but stimulated lysine accumulation [40]. who studied the effect of selected nutrients on lysine production from whey permeate by *Brevibacterium lactofermentum* ATCC 210 reported a lysine production of 3.3g/l when 0.2% yeast extract was added. They believed that yeast extract contain certain components that stimulate lysine production. Also in a study done by Yamada et al. [41] to study the effects of organic nitrogen sources on L-methionine production by *Methylotroph* strain OM 33, reported that 5mg/ml yeast extract stimulated the optimum yield of L-methionine. Chan and Foster [42] and Tauro et al. [43], reported a retardation in glutamic acid production by *Bacillus subtilis* and lysine accumulation in *Ustilago maydis*, respectively when growth promoters were used.

5. CONCLUSION

The study has shown that the addition of some vitamins, antibiotics and growth stimulators enhanced L-lysine production. However, *B. subtilis* PR13 produced the maximum yield of L-lysine. The supplementation of 0.1% v/v of peptone, 1 µg/ml of biotin and 0.04 units/ml of penicillin were optimal for L-lysine production by *B. subtilis* PR13.This development indicates that large scale L-lysine production is feasible in Nigeria and this will help to meet the present-day needs in its industrial sector.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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