Influence of Agitation rates, pH and Calcium carbonate on L-lysine Production from Agricultural Productsby *Bacillus* species Isolated From Nigerian Soil

ABSTRACT

L-Lysine is an essential amino acid that is required in the diet of humans and animals. It is utilized in human medicine, cosmetics and pharmaceutical industry. The influence of agitation rates, pH and calcium carbonate on L-lysine production from cheap agricultural products by *Bacillus* species isolated from Nigerian soil was studied. 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, biochemical and molecular characteristics.Optimization of some parameters which included agitation rates, pH values and $CaCO_3$ concentrations, on L-lysine production by the *Bacillus* species was carried out. The L-lysine was produced in 250 ml flasks containing fermentation media (FM1 and FM2). The findings revealed that, enhanced L-lysine yield of 2.10 and 1.33 mg/ml was observed at agitation rate of 180 rpm for *B. subtilis* PR13 andPR9 respectively. There was a positive correlation between agitation rates and L- lysine production by *B. subtilis* PR13 andPR9 (r = 0.96 and 0.83 respectively). The pH of 7.5, stimulated optimum L-lysine yield of 2.27 mg/ml for PR13 and 1.38 mg/ml for PR9. There was a positive correlation between pH values and L-lysine production by *B. subtilis* PR13 andPR9 (r = 0.63 and 0.50 respectively). The supplementation of 40g/l of $CaCO_3$, enhanced optimum L-lysine yield of 2.18 mg/ml for *B. subtilis* PR 13 and 1.30 mg/ml for *B. subtilis* PR9. There was a positive correlation between varying concentrations of calcium carbonate and L-lysine production by the *B. subtilis* PR13 (r =0.35), while negative correlation was observed for *B. subtilis* PR 9 (r = -0.10). The results obtained in the study illustrated that the

optimization of process parameters could increase the L-lysine yield from agricultural products by*B. subtilis* PR13 and *B. subtilis* PR9.

Keywords:*Bacillus* species, L-lysine, Submerged fermentation, Agitation rate, pH

1. INTRODUCTION

"Amino acids are molecules used by all living things to make proteins and they contain both amino and carboxylic acid functional groups. Amino acid fermentation has reached a stage where it is playing a vital role for the supply of natural amino acids at industrial level. The discovery of glutamic acid producing bacterium,*Micrococcus glutamicus* (later renamed as *Corynebacteriumglutamicum*) gave a new dimension to amino acid production. This break through laid the foundation for other researchers who lately reported many bacteria involved in amino acid fermentation" [1]. "Microbial fermentation provides 100% L-amino acids, whereas by chemical method, 50% D and 50% L- amino acids are obtained" [2]. "Amino acids are major industrial products derived by fermentation, covering a world market of more than 5 million tons per year" [3]."The most extensive formed amino acid (roughly 900,000 tons each year) occurs as L-glutamic acid, trailed by L-lysine (420,000 tons each year) as well as DL-methionine (350,000 tons each year) while the rest of the amino acids trail behind" [4].

"L-Lysine is one of the 9 amino acids which are essential for human and animal nutrition. It is not synthesized biologically in the body.L-lysine is useful as medicament, chemical agent, food material and feed additive" [5]."L-Lysine along with some other amino acids like aspartic acid is used extensively in the pharmaceutical industry in the formulation of diets with balanced compositions and in amino acid infusion" [6,7]. "As a fine chemical, it is utilized in human medicine, in cosmetics and as precursor for industrial chemicals. L- Lysine deficiency in man has been linked to the development of various diseases and

physiological conditions including anemia, nausea, dizziness, blood shot eyes, hypersensitivity to sound, impaired growth, hair loss, disorders of reproductive system, osteoroporosis, cystinuria, and immunodeficiency" [8,9].

"L-Lysine production is not restricted to any particular group of microorganisms, though the high yielding strains are mostly the species of *Arthrobacter*, *Corynebacterium*and *Brevibacterium"*[10]. "Very good producer strains have also been developed from *Bacillus subtilis*and *Escherichia coli"* [11].

"Both chemical and biochemical methods are used for L- lysine production" [5,12]. "From the commercially manufactured L-lysine, 80% is manufactured by biochemical method and only 20% by chemical means" [13]."Among biochemical methods, fermentation is the most economical and practicable means of producing lysine"[14]. "Its major commercial form is L-lysine-HCL (L-lysine monohydrochloride). Of course, each L-lysine commercial form requires different downstream processing to achieve the degree of purity (implying significant differences in investment costs, losses during down streaming, amount of waste volume, and user friendliness), so that L-lysine-HCl involves higher operating costs due to the larger number of equipment and energy consumption. 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" [17].

"As Nigeria is a developing country, a huge amount of foreign exchange is spent in the importation of L-lysine for our industries. There is huge potential in production of L-lysine locally by microbiological methods using available agricultural products. 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" [18]. In a further study, the *Bacillus* species were used for L-lysine production using carbohydrates as carbon and seed meals as nitrogen sources [19].

The present research work was aimed at determining the influence of agitation rates, pH and calcium carbonate on L-lysine production from agricultural products by *Bacillus* species (*B. subtilis* PR13 and *B. subtilis* PR9) isolated from Nigerian soil.

2.0 MATERIALS AND METHODS

2.1 Isolation of bacteria

The three bacterial isolates used in this study were isolated from different locations in Awka town, Anambra state, Nigerian [18]. They were purified and Identified as *B. subtilis* PR13 and *B. subtilis* PR9, using cultural, biochemical and molecular characteristics. The bacteria culturesare maintained at 4°C until used and examined for the production of L-lysine.

2.2 Inoculum preparation

Two loopfulsof *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 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 for 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 by enzyme hydrolysedpowdered millet (an agricultural product) 60g;the nitrogen source (ammonium

sulphate) was replaced by soyabean meal 40g ; water, 1 litre; pH 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 by enzyme hydrolysedpowdered sorghum (an agricultural product) 60g, the nitrogen source (ammonium sulphate) was replaced bydeffated peanut meal (an agricultural product) 40g; water, 1 litre; pH adjusted to 7.2 was used for *Bacillus subtilis* PR9.

2.4Optimization of culture conditions for L-ysine production

2.4.1 Effect of agitation rates

The effect of the different agitation rates on growth and lysine production by *B. subtilis* PR13 and PR9 were 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. 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 different agitation rates (120, 140, 160, 180 and 200 rpm) and incubated at 30^{o}C for 72h. 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 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

2.4.2 Effect of pH values

The effects varying pH values on growth and L-lysine production by *B. subtilis* PR13 and PR9 were studied. A (100ml) Erlenmeyer flasks containing 20 ml fermentation media (FM1 and FM2) as was previously described was used for lysine production. The media were prepared at different pH values of 6.5, 6.8, 7.0, 7.2, 7.5 and 8.0 and sterilized at 121^{o}C for 15 min.

After sterilization, the media were cooled to room temperature and 1ml (1.8×10^{7}cfu /ml) 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 72 h. Thereafter, residual sugar, bacterial growth and L-lysine production were determined from the broth culture as was previously described. The experiments were conducted in triplicate.

2.4.3 Effect of calcium carbonate

The effects of different concentrations of calcium carbonate on growth and L-lysine productionwere 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 concentrations of calcium carbonate (20, 30, 40, 50 and 60g/l) were added to the fermentation media and sterilized at 121^{o}C for 15min. After sterilization, the media were cooled to room temperature and 1ml (1.8×10^{7}cfu/ml) 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 as was previously described. 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[20]. 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 [21]. 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 12ml with distilled water. After allowing the reaction mixture to stand for 15min at room temperature, the optical density was measured at 540nm 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

3.0 RESULTS

The effect of agitation rates on growth and lysine production by *B. subtilis* PR13 andPR9 are presented in Figures 1-2. The results showed that maximum growth and lysine yields by *Bacillus subtilis* PR13and *Bacillus subtilis* PR9, were observed at agitation rate of 180 rpm. The highest lysine accumulation of 2.10 and 1.33 mg/ml by *B. subtilis* PR13 and PR9, corresponded with a residual sugar of 0.60 and 0.53 mg/ml respectively. There was a positive correlation between agitation rates and lysine production by *B. subtilis* PR13 andPR9 (r = 0.96 and 0.83 respectively)

The result of the effect of pH on growth and lysine production by *B. subtilis* PR13 and *B. subtilis* PR9 are presented in Figures 3-4.The results show that maximum growth and lysine yields by the *Bacillus* strains, were observed at a pH 7.5. The highest lysine accumulation of 2.27 and 1.38 mg/ml by*B.* PR13 and PR9 respectively, corresponded with a residual sugar of 0.45 and 0.51 mg/ml respectively. There was a positive correlation between pH values and lysine production by *B. subtilis* PR13 andPR9 (r = 0.63 and 0.50 respectively).

The effectof calcium carbonate on growth and lysine production by *B. subtilis* PR13 and PR 9 are presented in Figures 5-6.The results showed that maximum growth and lysine yields by all the *Bacillus* species, were observed when 40g/l of $CaCO_3$ was added. The highest lysine accumulation of 2.18 and 1.30mg/ml by *B.subtilis*PR13 and PR9 respectively, corresponded with residual sugars of 0.33 and 0.50 mg/ml respectively.There was a positive correlation between varying concentrations of calcium carbonate and lysine production by the *B. subtilis* PR13 (r =0.35), while there was negative correlation for *B. subtilis* PR 9 (r = -0.10)

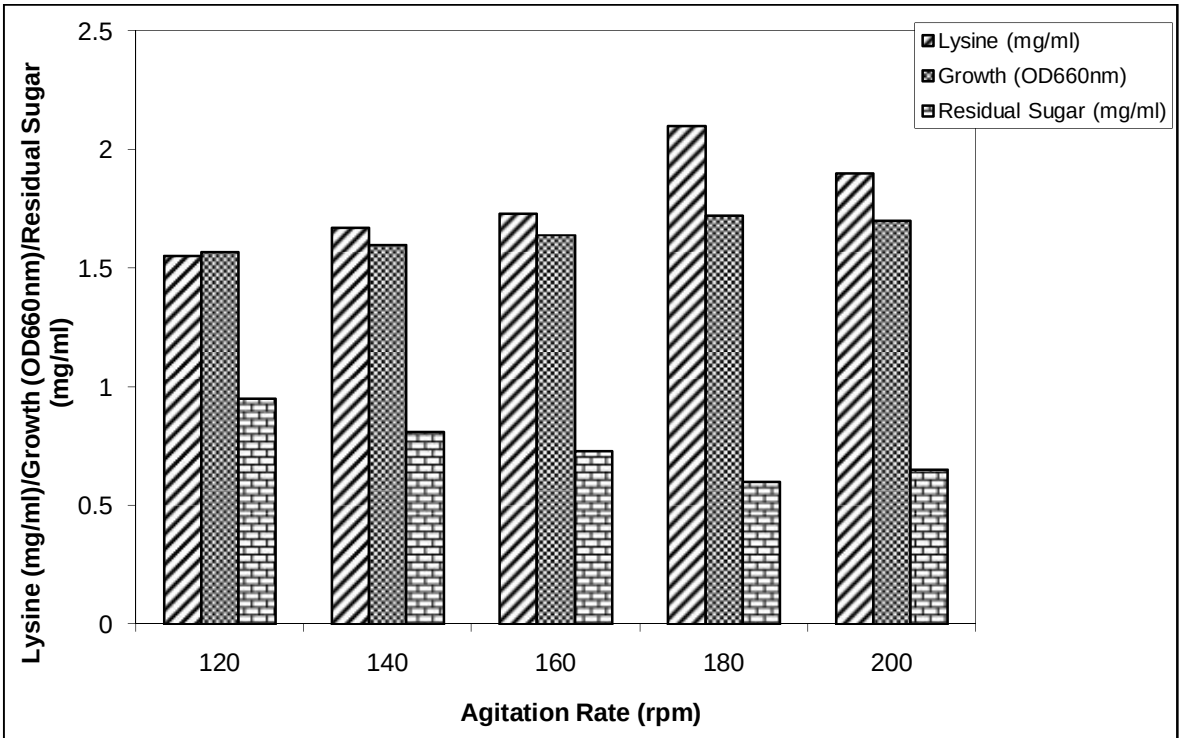

Figure 1: Effect of Agitation Rates on Lysine Production by *Bacillus subtilis* PR13

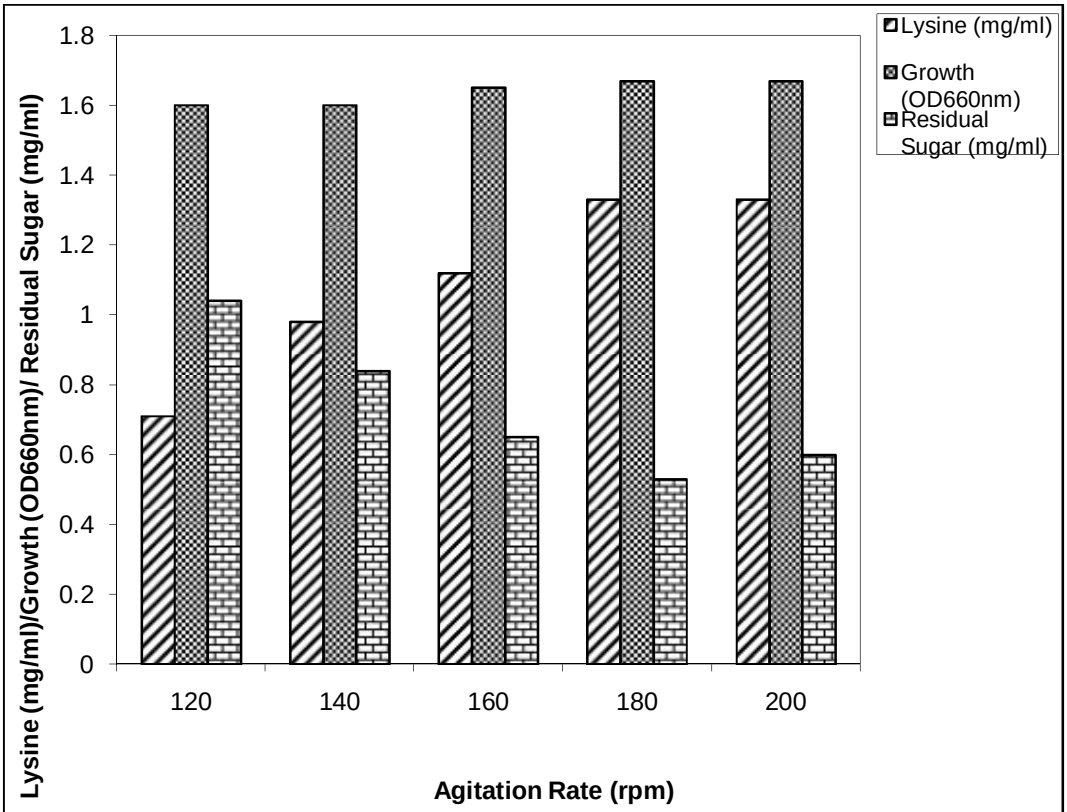

Figure 2: Effect of Agitation Rates on Lysine Production by *Bacillus subtilis* PR9

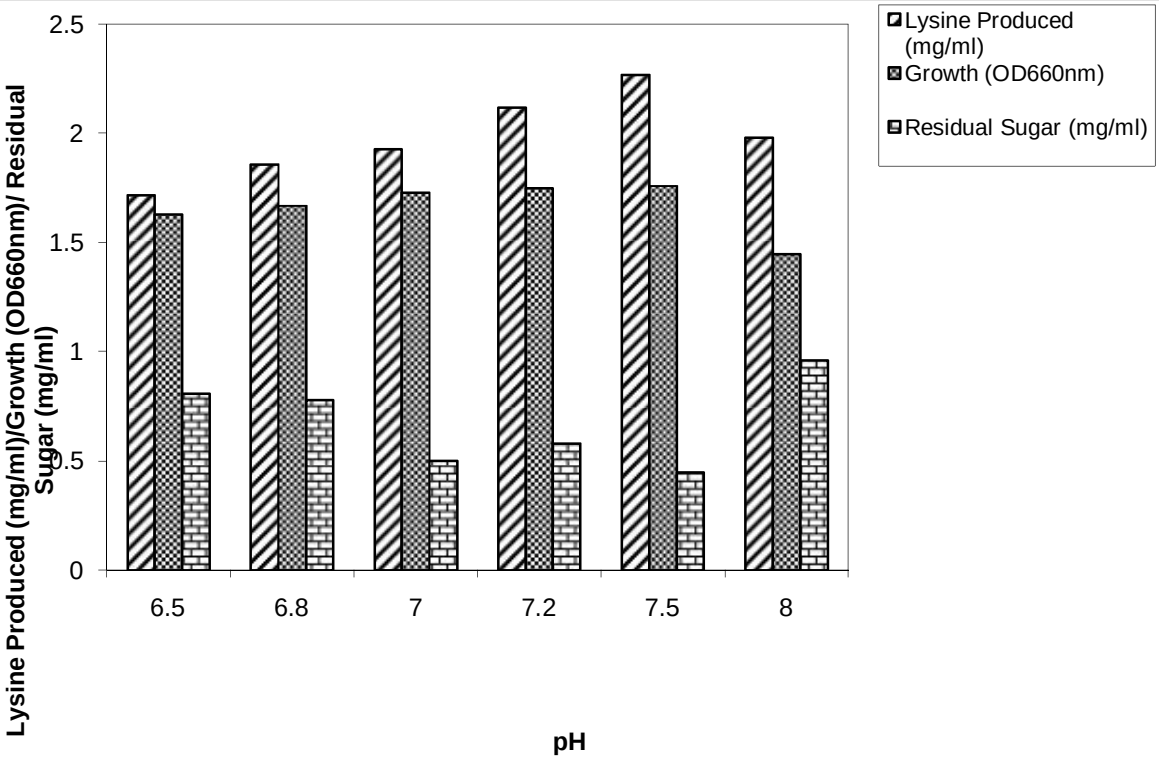

Figure 3: Effect of pH Values on Lysine Production by *Bacillus subtilis* PR13

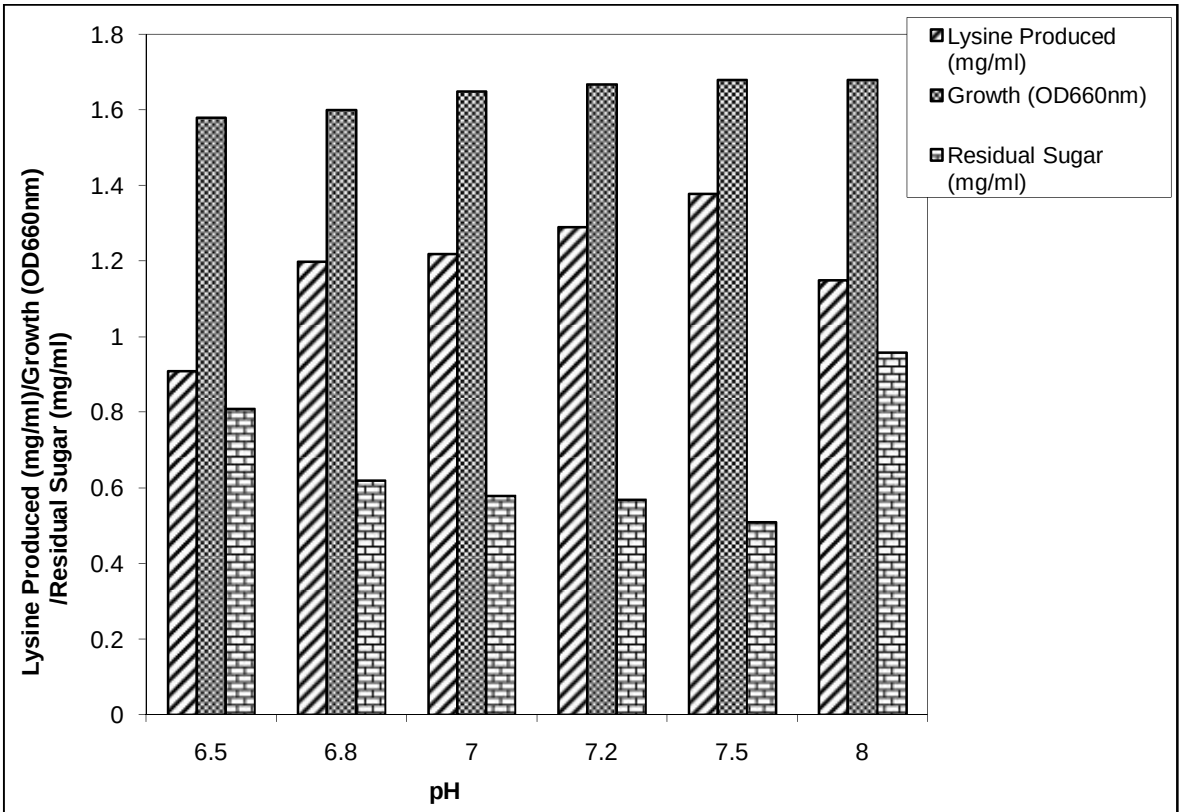

Figure 4: Effect of pH Values on Lysine Production by *Bacillus subtilis* PR9

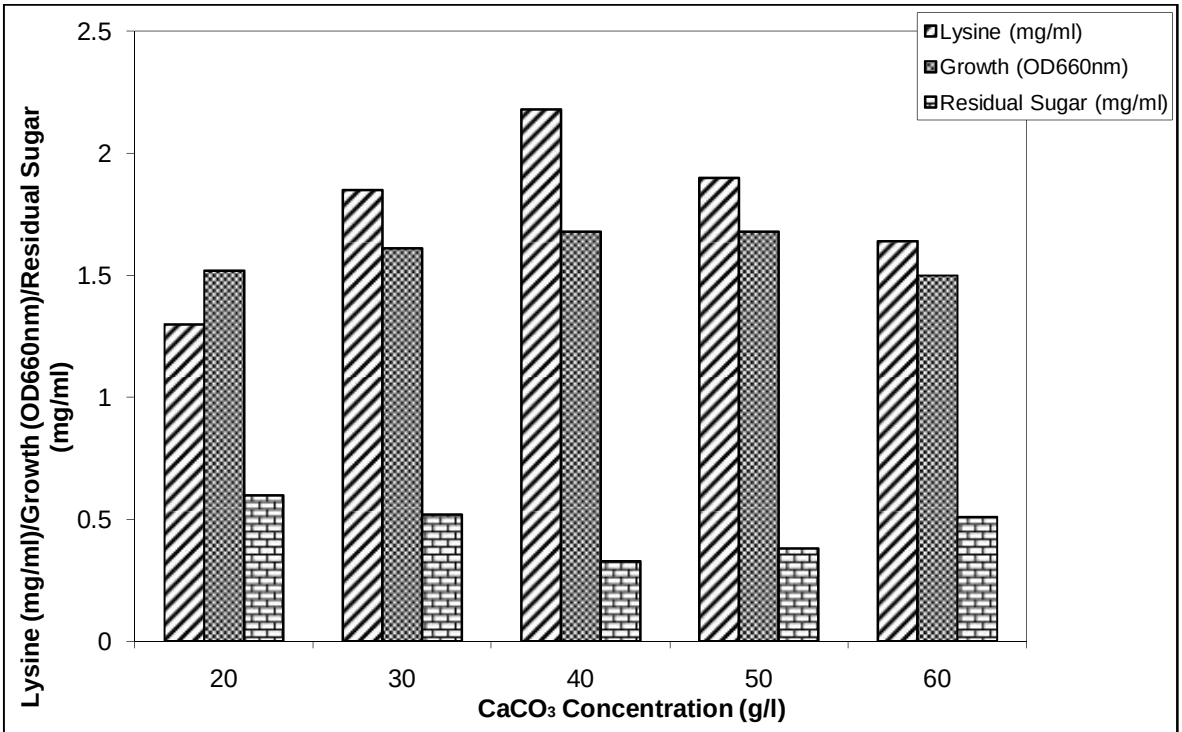

Figure 5: Effect of Calcium Carbonate on Lysine Production by *Bacillus subtilis* PR13

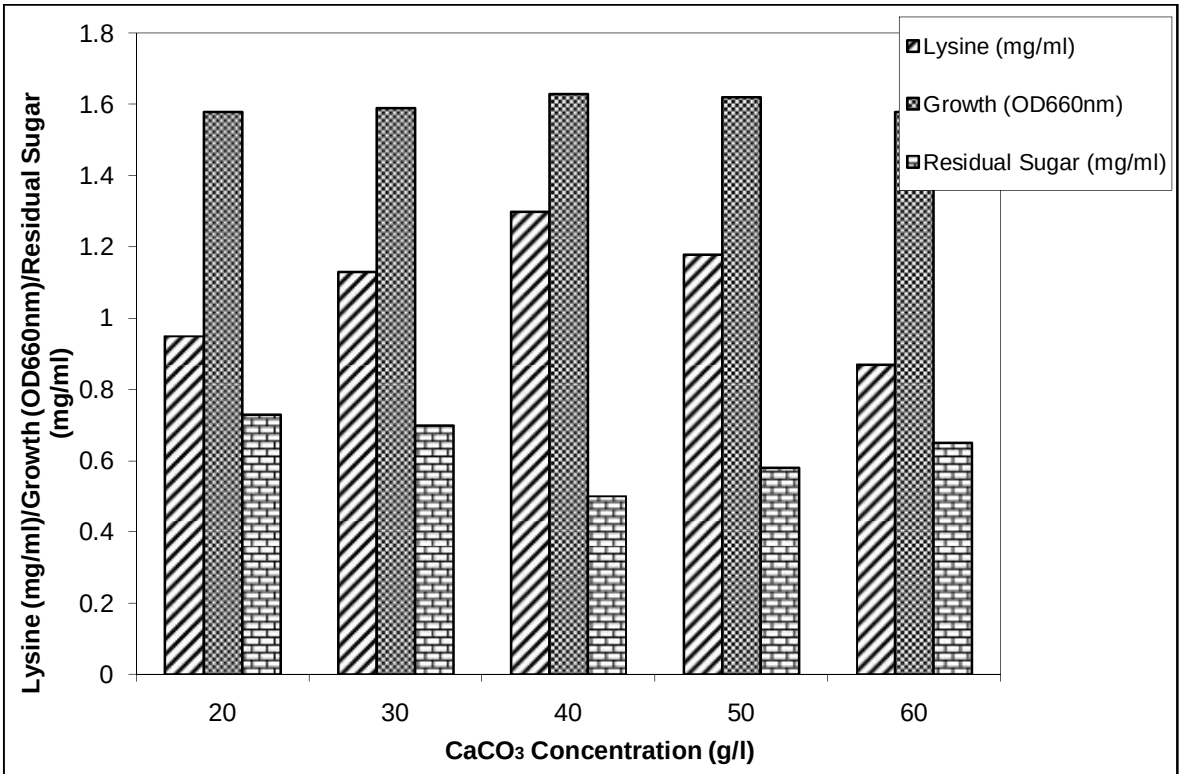

Figure 6: Effect of Calcium Carbonate on Lysine Production by *Bacillus subtilis* PR9

4.0 DISCUSSION

The study revealed that maximum L-lysine yield by *B. subtilis* PR13 and *B. subtilis* PR9 was observed at agitation rate of 180rpm, which later decreased. This was contrary to the findings of Shah et al. [22] who studied "the effect of agitation on lysine production by *Corynebacteriumglutamicum*. They observed that L- lysine production increased from 50rpm to the maximum at 200rpm". Also, Wang et al. [23] worked "at 200 rpm for fermentation of L-lysine on a rotator shaker". Rehman et al.[24] reported that "L-lysine production increased from 12.5 mg/mL to 15.1 mg/mL in shake flask cultures when agitation rate was increased from 150 to 300 rpm". Siripoke et al. [25] reported "an increase in L- lysine production when the agitation rates were increased from 300 to 400 rpm, however L-lysine yield decreased when the agitation rate was increased to 450rpm". They opined that the decrease in L-lysine production may be due to the increasing foam observed which has resulted in the reduction of the oxygen transfer rate to the cells. Lee et al.[26] suggested that "agitation rates above

200rpm will lead to denaturation of enzymes with attendant low production of metabolites". Darah and Ibrahim [27] pointed out that "excessive agitation could lead to cell lysis and increase cell permeability due to abrasion by shear forces". Shafee et al.[28] pointed out that "mixing is especially important because oxygen is a very low solubility nutrient. The oxygen transfer capability of flask can limit the amount of biomass that can be grown in the flask. When premature oxygen limitations are imposed on growth, changes in physiological state occur which results in reduced inoculum effectiveness". Oxygen is essential for growth and yields of metabolites in aerobic organism. For the oxygen to be absorbed it must be dissolved in aqueous solution along with the nutrients by agitating with rotary shaker. Agitation is done to provide homogeneity. Agitation intensity influences the mixing and oxygen transfer rate in many bacterial fermentations thereby influencing growth and product formation. "Under aerobic conditions, sufficient oxaloacetate is available for aspartate conversion and then flows towards L- lysine formation. However, under anaerobic conditions, carbon metabolism shifts towards formation of ethanol, lactic acid and succinate"Wendisch et al. [29].

The result of the study showed that maximum L-lysine yield was observed at pH 7.5 for both *B. subtilis* PR13 and *B. subtilis* PR9. This is contrary with the report ofSiripoke et al. [25] who reported maximum lysine production of 6.73g/l for *Bacillus* SWU41, at a pH 6.0, unlike most lysine producing strains which prefer pH 7.0–7.5. [30] reported maximum lysine yield of 69.5g/l at pH 7.0 using *Brevibacteriumflavum*, above pH 7.5 the sugar utilization and L-lysine production slightly decreased.Hua et al. [31] in a study of external pH on lysine production by *Corynebacteriumglutamicum* observed that the optimum pH for lysine flux was found to be 7.0.Liu [32] found that L-lysine synthesis decreased at pH lower than 6.5, but found no difference between 6.5 and 8.0. An initial pH 7.5 was recommended by L-lysine production by *Corynebacteriumglutamicum*Broer and Kramer[33].Rehman et al. [24] observed that at pH value of 7.6, optimum production of L-lysine (12.5 mg/mL) in basal medium was in agreement with the results ofBroer and Kramer [33] and Hua et al [31].Broer et al. [34] found that the optimum pH for maximum velocity of

transport by *Corynebacteriumglutamicum* was 7.4 – 7.8.Kelle et al.[35] studied "the effect of pH on L-lysine transport by *Corynebacteriumglutamicum* and found that pH value governed the transport activity and the specific L-lysine export rate. Membrane potential was found to be an important factor influenced by pH of fermentation medium towards lysine excretion by the cells"**.**

The results from the study revealed that 40 g/l of $CaCO_3$ was optimum for lysine production by the *Bacillus* species. This is in contrast to with the report of Shah et al. [22] that at 2% CaCO3 *Corneybacteriumglutamicum* produced maximum yield of L-lysine (16.58g/l). Ekwealor and Obeta[36], Rao et al. [37] andSiripoke et al. [25] incorporated different concentrations of CaCO3 in the fermentation medium during the production of lysine by *Bacillusmegaterium*, *Corneybacteriumglutamicum* and *Bacillus* SWU 41 respectively. Young and Chipley [38] reported that the addition of CaCO3 with yeast extract resulted in a relatively constant pH and increased depletion of glucose, but reduced lysine production. "Decrease in pH could be due to accumulation of acids such as pyruvic acid, lactic acid, gluconicacid as a result, bacterial growth ceased with concomitant decrease in the product yield. pH is one of the most important factors affecting microbial propagation"[1]. "As nutrients are consumed and converted into product during the fermentation process, the pH changes markedly in the absence of suitable control mechanism. In order to maintain optimal pH, reagents like Calcium carbonate must be added to the culture medium at the beginning of the fermentation"[1]. Calcium carbonate neutralized the pH of the broth, acting as internal neutralizing or buffering agent, it would be useful for shortening the fermentation time. Wang et al. [39] reported that though the pH of the fermenter was automatically controlled by ammonia water, but still small amount of $CaCO_3$ must be added. It eliminates the lag phase of cell growth, thereby shortening fermentation time. The free hydrogen ions are

consumed resulting in decreased hydrogen ion activity. In other words calcium carbonate acts to neutralize or buffer the solution by consuming hydrogen ions

5. CONCLUSION

The study revealed that some agricultural products could be used for L-lysine production. It also revealed that there was improved L-lysine production by *B. subtilis* PR13 and *B. subtilis* PR9 during the optimization study which included, agitation rates, pH and calcium carbonate.The agitation rate of 180 rpm, pH 7.5 and calcium carbonate 40g/l were optimum for L-lysine production. However, the highest L-lysine yield was observed with *Bacillus subtilis* PR13. The*Bacillus*species (*Bacillussubtilis*PR 13 and PR9) have shown potential for lysine production using readily available agricultural products. This development indicates that they can be used for large scale production of the enzyme, to meet present-day needs in the Nigeria's industrial sector. The microbiological process of L-lysine production, if well developed could lead to the availability of the product in Nigeria and this to some extent will reduce the importation of the product into the country. Further research is needed to study the effect of other parameters on L- lysine production.

COMPETING INTERESTS

Authors have declared that no competing interest exist

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