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The influence of surfactants and bivalent metals on L-lysine production by *Bacillus subtilis* using agricultural products as carbon and nitrogen sources

J. Okpalla [1, *] and I. A. Ekwealor [2]

[1] Department of Microbiology, Chukwuemeka Odumegwu Ojukwu University, P.M.B. 02 Uli, Anambra State, Nigeria.
[2] Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.

Abstract

The influence of surfactants and bivalent metal ions 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 *B. subtilis* PR13 and *B. subtilis* PR9, using cultural and biochemical characteristics. Optimization of some parameters which included, surfactants and bivalent metal on L-lysine production by *B. subtilis* 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 3.30 mg/ml and 1.46 mg/ml by *B. subtilis* PR13 and *B. subtilis* PR9, was observed in the presence of 0.1 µg/ml of stearic acid and 0.1% v/v Tween 80 respectively. The supplementation of 5 µg/ml of $CuSO_4$ and 1 µg/ml of $NiCl_2$, enhanced optimum L-lysine yield of 3.18 mg/ml for *B. subtilis* PR 13 and 1.51 mg/ml for *B. subtilis* PR9 respectively. The results obtained in the study illustrated that the optimization of process parameters could increase the L-lysine yield by *B. subtilis* PR13 and *B. subtilis* PR9.

Keywords: *Bacillus* species; L-lysine; Submerged fermentation; Surfactants

1. Introduction

Amino acids are the basic building blocks of proteins, which are the most important macromolecules for human and animal functions. The discovery of glutamic acid producing bacterium, *Micrococcus glutamicus* (later renamed as *Corynebacterium glutamicum*) 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].

Out of the 20 L-amino acids ecumenically found in most of living organisms, L-lysine is one of the 9 amino acids which are essential for human and animal nutrition [2]. It is used as food supplements for humans (children have a high requirement of lysine, since it is needed for bone formation) [3, 4]. It is used to formulate an amino acid balanced diet and in amino acid infusions [3]. It plays an essential role in the production of carnitine, a nutrient responsible for converting fatty acids into energy and helping to lower cholesterol. It also helps the immune system ward off viral infections, like herps, cold sore, mouth ulcers and the associated fever [5].

Lysine can be produced in different ways including chemical synthesis, extracting from protein hydrolyzate, enzymatic method, fermentation method, protoplast fusion technique and recombinant DNA technology [6,7]. Among these methods, fermentation is the most economical and practical means of producing lysine, as in this method low temperature, low pressure and low-cost carbon sources are used and a biological form of lysine (L-lysine) is produced [8].

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* Corresponding author: J. Okpalla; Email: judyzuby@yahoo.com

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]. Utilization of agricultural by-products as substrates for fermentation might offer an inexpensive alternative for microbial products such as amino acids. For industrial fermentations, the use of complex sugar substrates such as cane molasses, beet molasses, or hydrolysates from corn, wheat or cassava became standard. The type of the sugar used depends on the geographical location of the production plant [4,12,2]. As nitrogen sources, various inorganic and organic salts and compounds such as ammonium salts and other similar compounds, urea, natural proteolytic organic substances such as peptone, casein hydrolysate, yeast extract, corn steep liquor, soybean protein hydrolysate, and various other extracts of vegetal and animal tissues may be employed. In addition to carbon and nitrogen sources, the culture media employed for production of this amino acid, normally contains usual inorganic nutrients and essential elements for growth of micro-organisms [13]. Several researches have been carried out using agricultural products in production as basic carbon and nitrogen sources [14,15,16].

As the need for L-lysine increases for industrial processes in Nigeria, there is need to develop a cheap microbiological method for L-lysine production using agricultural by-products. Because of the availability of these agricultural products in Nigeria, lysine production by fermentation process may likely be more economical. Nigeria is a developing country, a huge amount of foreign exchange is utilized in the importation of this essential amino acid for the local industries. Nutritional and physical parameters affect the growth and product yield of organism [17,18]. Since each bacterium has definite range of culture conditions for better growth and for high production of L-lysine, therefore it is essential to investigate the effects of cultural conditions on bacterial growth and product yield.

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 [19]. In another study, the *Bacillus* species were used for L-lysine production using carbohydrates as carbon and seed meals as nitrogen sources [20]. The present study was aimed at investigating the influence of surfactants and bivalent metal ions on L-lysine production by *Bacillus subtilis* using agricultural products as carbon and Nitrogen sources

2. Material and methods

2.1. Microorganisms and culture maintenance conditions

Bacillus subtilis PR13 and *B. subtilis* PR9 isolated from different soil [15] 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 °C. Thereafter, the cultures were then preserved at 4 °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, 10.0 g; yeast extract, 10.0 g; NaCl, 5.0 g; water, 1litre; pH was adjusted to 7.2. Two loopful of *B. subtilis* PR13 and PR9 were inoculated into an Erlenmeyer flask containing 50 ml of seed medium which had already been sterilized at 121 °C for 15 min. The inoculated flasks were incubated for 24 h on a rotary shaker at 120 rpm and 30 °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 20 ml of fermentation medium 1 (FM1). The medium , was composed of KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.001 g; $MnSO_4.H_2O$, 0.001 g; $FeSO_4.7HO$, 0.001 g; $CaCO_3$, 50 g, the carbon source (glucose) was replaced with millet starch hydrolysate 60g; the nitrogen source (ammonium sulphate) was replaced with soyabean meal 40 g; water, 1 litre; pH was adjusted to 7.2. For *Bacillus subtilis* PR 9, L-lysine production was done in 100 ml Erlenmeyer flasks, containing 20 ml of fermentation medium 2 (FM2). The medium, was composed of KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.001 g; $MnSO_4.H_2O$, 0.001 g; $FeSO_4.7HO$, 0.001 g; $CaCO_3$, 50 g, the carbon source (glucose) was replaced with sorghum hydrolysate 60 g, the nitrogen source (ammonium sulphate) was replaced with deffated peanut meal 40 g; water, 1 litre; pH was adjusted to 7.2. The carbon source substrates were prepared in the laboratory using the method of Umerie *et al.* [15].

2.4. Optimization of culture conditions for L-lysine production

2.4.1. Effect of surfactants

The effect of surfactants, which included 0.01 – 1.00 µg/ml of palmitic acid and stearic acid and 0.05 – 0.50 % v/v of oleic acid, Tween 80 and linoleic acid on L-lysine production was determined. Fermentation was carried out in 100 ml Erlenmeyer flasks containing the 20 ml of fermentation media FM1 and FM2 as was previously described. The surfactants were added to the fermentation media and sterilized at 121°C for 15 min. One milliliter volume (1.8×10^7 cfu/ml) of 24 h 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, 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 bivalent metals

The effect of bivalent metals, which included 0.10-10.0 µg/ml of $ZnSO_4$, $NiCl_2$, $CuSO_4$, $CaCl_2$ and $CoCl_2$ on L-lysine production was determined. Fermentation was carried out in 100 ml Erlenmeyer flasks containing the 20 ml of fermentation media FM1 and FM2 as was previously described. The bivalent metals were added to the fermentation media and sterilized at 121°C for 15 min. One milliliter volume (1.8×10^7 cfu/ml) of 24 h 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, 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. Quantitative determination of lysine

L-lysine in the broth culture was determined by acidic ninhydrin method of Chinard [21]. A 5 ml volume of the culture broth of the isolate was centrifuged at 5000 ×g for 20 min, and the cell-free supernatant was collected and assayed for lysine production. One milliliter (1 ml) of glacial acetic acid was added to 1 ml of supernatant in a test tube. Thereafter, one ml of a reagent solution which contains an acid mixture, 0.4 ml of 6 M orthophosphoric acid, 0.6 ml of glacial acetic acid and 25 mg of ninhydrin, was also added to the supernatant in the test tube. The blank contains 1 ml of glacial acetic acid, 1 ml of the acid mixture without ninhydrin and 1 ml supernatant. Both tubes were capped and the contents mixed properly for 10 min before heating at 100 °C in a water bath for 1 h. 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 5 ml.The optical density of the reacting mixture was read against the blank at 515 nm in a spectrophotometer. Results obtained with the test samples were extrapolated from a standard lysine curve.

2.5. Estimation of reducing sugar

The reducing sugar content was determined by dinitrosalicyclic acid (DNS) method of Miller [22]. Reducing sugar was estimated by adding 1 ml of DNS to 1 ml of the supernatant. The mixture was heated in a water bath at 100 °C for 10 min 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 15 min 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.

2.6. Statistical analysis

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

3. Results

The results of the effect of surfactants on lysine production by *Bacillus subtilis* PR13 and *B. subtilis* PR9 are shown in figures 1 and 2. The highest L- lysine production of 3.3 and 1.46 mg/ml by *Bacillus subtilis* PR13 and *B. subtilis* PR9 was observed at the supplementation of 0.1 µg/ml of stearic acid and 0.1 % v/v of Tween 80 respectively. There was a negative correlation between stearic acid and lysine production by *B. subtilis* PR13 (r= -0.50), while positive correlation existed between Tween 80 and lysine production by *B. subtilis* PR9 (-0.42).

The results of the effect of bivalent metals on lysine production by *B. subtilis* PR13 and PR 9 are presented in figures 3-4. The results showed that maximum lysine yields of 3.18 and 1.51 mg/ml by *Bacillus subtilis* PR13 and PR9, were

observed at the addition of 5 µg/ml of $CuSO_4$ and 1 µg/ml of $NiCl_2$ respectively. There was a negative correlation between CuSO4 and lysine production by the *B. subtilis* PR13 (r=-0.30), while negative correlation existed between $NiCl_2$ and lysine production by the *B. subtilis* PR9 (r= -0.77).
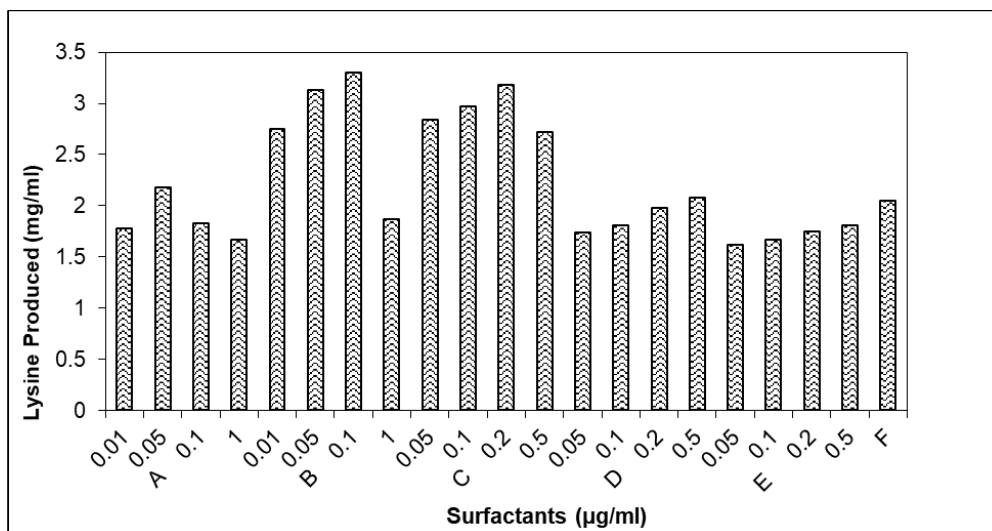

Figure 1 Effect of Surfactants on L-lysine Production by *Bacillus subtilis* PR13: A, palmitic Acid ; B, Stearic acid C, Oleic acid (%v/v); D, Tween80 (%v/v); E, Linoleic Acid (%v/v); F, Control (without surfactants)
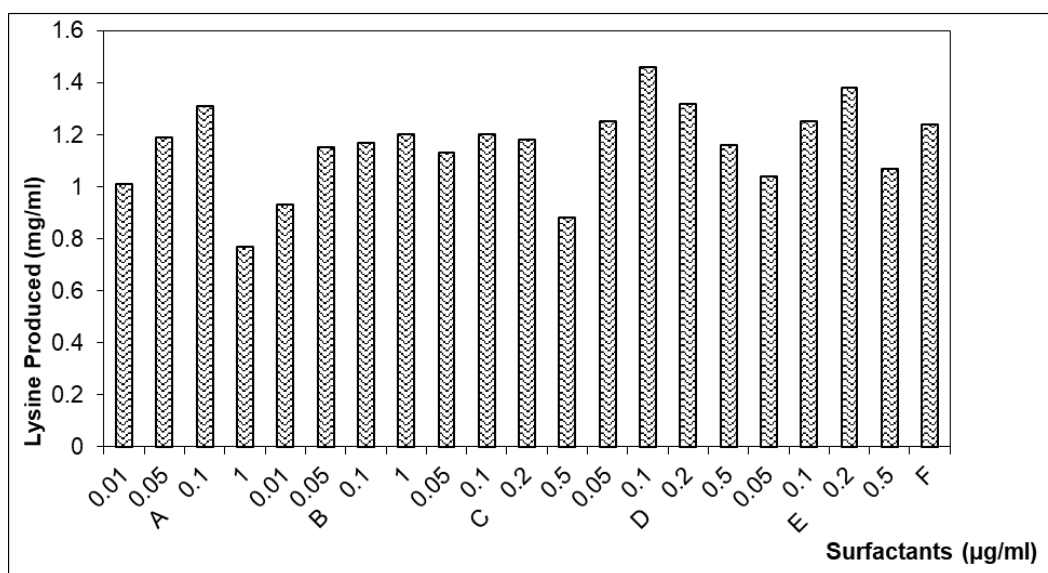

Figure 2 Effect of Surfactants on L-lysine Production by *Bacillus subtilis* PR9: A, palmitic Acid ; B, Stearic Acid ; C, Oleic Acid (%v/v);D, Tween80 (%v/v); E, Linoleic Acid (%v/v); F, Control (without surfactants)
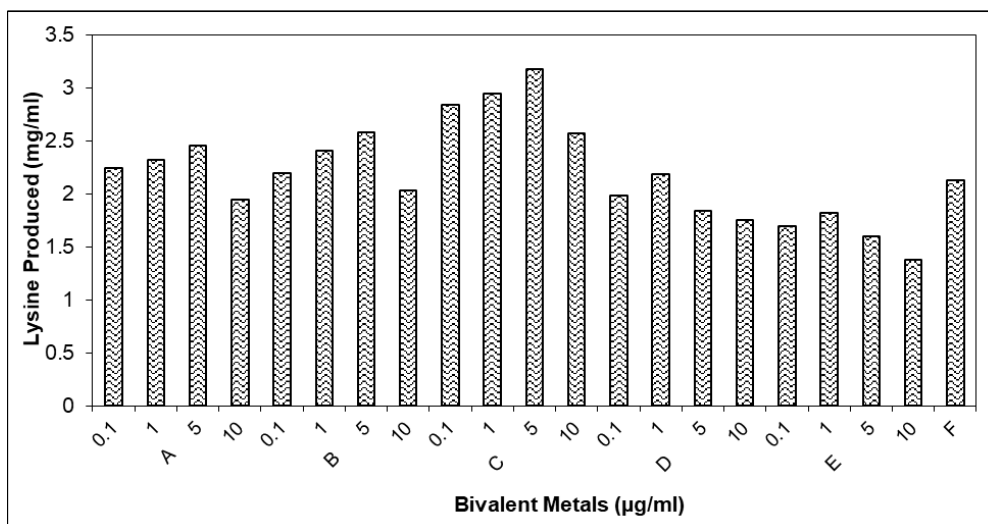
Figure 3 Effect of Bivalent Metals on L-lysine Production by *Bacillus subtilis* PR13: A, ZnSO$_4$; B, NiCl$_2$; C, CuSO$_4$; D, CaCl$_2$; E, CoCl$_2$; F, Control (without bivalent metals)
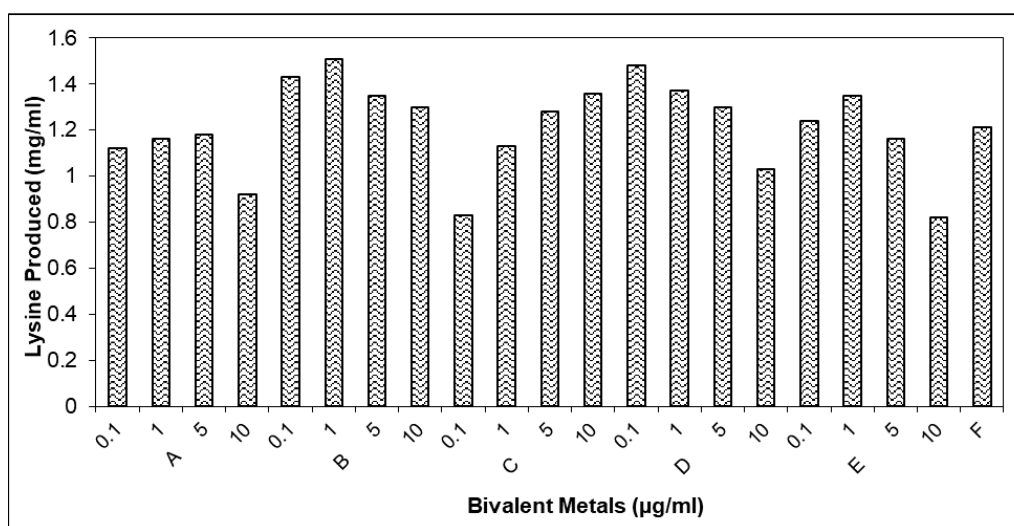

Figure 4 Effect of Bivalent Metals on L-lysine Production by *Bacillus subtilis* PR9 A, ZnSO$_4$; B, NiCl$_2$; C, CuSO$_4$; D, CaCl$_2$; E, CoCl$_2$; F, Control (without bivalent metals).

4. Discussion

Surfactants decrease surface tension and increase the air supply of the medium. Various kinds of surface active agents are known to affect permeability in microorganisms [23,24]. Tween 80 stimulated improved lysine production by *B. subtilis* PR9. This is in agreement with Konicek *et al.* [25], who studied the effect of Tween80 on biosynthesis of L-lysine in regulatory mutants of *Corynebacterium glutamicum*. They observed that by using Tween80, the production of L-lysine was increased by 23-25 % respectively. The stimulation observed is supposed to be caused by influencing cellular surface structure. Smekel *et al.* [26], tested the effect of several types of polar and non- polar tensides on the biosynthesis of L-lysine using the *Corynebacterium glutamicum*. Only the definite concentrations of liquid Tween 60 and 80 had a stimulating effect on the production of L-lysine. In contrast, Zaki *et al.*[27] reported the production of amino acids by *Bacillus ammoniagenes* and observed that in the presence of Tween 80 lesser amount of lysine was produced. Oleic acid stimulated enhanced lysine production in *Bacillus subtilis* PR9. This is in contrast with the finding of Ekwealor and Obeta [28], who noted that oleic did not encourage lysine production in *Bacillus megaterium*. Zaki *et al.* [27] reported that in the presence of 100-169mg% sodium oleate, 100-169mg% lysine was produced in *Brevibacterium ammoniagenes.* In the study, linoleic acid did not stimulate L- lysine production in *B. subtilis* PR13 and *B. subtilis* PR9. This is contradictory

to the report of Ekwealor and Obeta [28] who reported a stimulating effect of linoleic acid on lysine production by *Bacillus megaterium.* Results from the study showed that palmitic acid (0.1 μg/ml) stimulated enhanced lysine yield in *Bacillus subtilis* PR9 and is in line with the work of Takinami *et al.*[29].They reported that palmitic acid stimulated the production of glutamic acid by a glutamic acid producing bacterium.

Results observed from the effect of bivalent metals, showed that $NiCl_2$ stimulated maximum lysine production in *Bacillus subtilis* PR9. This is in line with the report of Ekwealor and Obeta [30], who reported the stimulation of lysine production by Ni^{2+} in *Bacillus megaterium*. Thus, establishing the role of Ni^{2+} as an essential metal for several enzyme-catalyzed reactions in microorganisms as observed by Lancaster [31]. Welward *et al.*[32] and Sen and Chatterjee [9] in contradiction reported the inhibitory effect of Ni^{2+} on lysine accumulation by *Micrococcus glutamicus* and *M. varians* 2fa, respectively. According to Welward *et al.* [32] this may be as a result of the inhibition of diaminopimelic acid decarboxylase activity of the microorganisms by the metal ion. $CuSO_4$ and $CaCl_2$ stimulated lysine production in *Bacillus subtilis* PR13 and *Bacillus pumilus* PR9. Their role in product formation in microorganism is not yet known. Ekwealor and Obeta [30] reported that they increased lysine production in *Bacillus megaterium*. Harol (1986) pointed out that the role of Ca^{2+} in growth and product formation in microbes is not yet known but has been implicated in the stabilization of cell wall, activation of extracellular enzymes and in the regulation or triggering of a range of cell functions. Martin and McDaniel [34] and Hughes and Poole [35] highlighted that the metal ions probably act as activator or inhibitors of enzymes involved in the synthetic steps of metabolites. Zn^{2+} stimulated L- lysine production in *Bacillus subtilis* PR13, which is similar to the findings of Ekwealor and Obeta [30] who observed increased lysine production by Zn^{2+} in *Bacillus megaterium*. Sen and Chatterjee [9] reported the possible role of zinc in lysine production. Weinberg [36] noted the importance of the metal in growth of certain microorganisms, Sigel [37] reported its role in the synthesis of industrially and medically significant microbial secondary metabolites.

5. Conclusion

The study showed that some agricultural products could be harnessed as good substrates for L-lysine production by submerged fermentation. In the study, it was observed that, *B. subtilis* PR13 produced the maximum yield of L-lysine. The supplementation of 0.1 μg/ml of stearic acid and 5 μg/ml of $CuSO_4$ were optimal for L-lysine production by *B. subtilis* PR13. 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 Nigeria's industrial sector.

Compliance with ethical standards

Disclosure of conflict of interest

There is no conflict of interest to declare.

Authors' contributions

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

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