WWJMRD 2019; 5(4): 10-18
www.wwjmrd.com
International Journal
Peer Reviewed Journal
Refereed Journal
Indexed Journal
Impact Factor MJIF: 4.25
E-ISSN: 2454-6615

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Screening for Lysine Production by Bacteria Isolated From Nigerian Soil

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Abstract
Lysine production by bacteria isolated from Nigerian soil was studied. Bacterial isolates (460) from different soil samples in Awka town were screened for lysine production on solid agar medium seeded with lysine auxotroph *Escherichia coli.* The agar plates were observed for halo growth of the *E.coli* which indicates lysine production by the isolate. A total of 37 bacterial isolates were recovered as lysine producers with isolates HS26, PR13, SS67, PR29, PR9, HS60 and SS16 producing high lysine yields. The 37 isolates were further grown in submerged medium and 3 of the isolates identified as *Bacillus subtilis* PR 13, *B. subtilis* PR9 and *B. pumilus* SS16 were chosen as the best lysine producers. The maximum lysine yields for *Bacillus subtilis* PR13 and Bacillus *subtilis* PR9 were obtained at 20ml medium volume, while *Bacillus pumilus* SS16 accumulated highest lysine yield in a medium volume of 25ml. Highest lysine accumulation was achieved by *Bacillus subtilis* PR13 and Bacillus *pumilus* SS16 at 2ml of 10%, while *Bacillus subtilis* PR9 accumulated the highest lysine yield at 2ml of 8% inoculum size. The result of the study indicated that lysine producing bacteria were present in the soil.

Keywords: Lysine production, Bacteria, Soil, Screening, Lysine auxotroph

1. Introduction
The Lysine (abbreviated as lys or k) is an essential amino acid that is required in the diet of humans and meat producing animals (Jose *et al*., 2005). It is not synthesized biologically in the body. Its major commercial form is L-lysine-HCL (L-lysine monohydrochloride). L-lysine is commonly produced in a stable and non-hygroscopic hydrochlorinated form of a purity higher than 98.5% and moisture content less than 1% (Anastassiadis, 2007).In 2001, the world market for lysine was 550,000 tons with a growth rate 7% per year. It is used as food supplements for humans (children have a high requirement of lysine, since it is needed for bone formation) (Shah *et al*, 2002; Hermann, 2003).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 (Shah *et al.*, 2002) and as precursor for the synthesis of peptides or agrochemicals (Zelder *et al*., 2005).
Lysine defiency 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 (Meister,1965; Chen *et al*., 2003). To prevent the development of protein deficiency disease, amino acid supplementation is a prerequisite. Excess of lysine in the body can elevate the calcium levels which can lead to gallstones and obstructive diseases, and can also disrupt the process of protein synthesis in the body (Meister, 1965). In animals, it is used as a feed additive to enhance their nutritional value. Since livestocks are unable to synthesize this amino acid, it must be added to their feeds such as cereals which do not contain sufficient levels of L-lysine (Kreutzer *et al*, 2004). L-lysine is the first limiting amino acid absent virtually in every cereal grain known to man (How *et al*., 1965). It is added to improve the nutritional value of cereal-based diets and it is currently sold at approximately US$ 3-4/kg the recommended nutrient intake of lysine for normal adult is 1.6g/day. Presently 80% of lysine in the world market is made by microbial fermentation and the remaining 20% by chemical synthesis (Siripoke, 2005;

Anastassiadis, 2007).When compared to chemical methods, fermentation production has the advantage of yielding the optically active and biologically required L-form of amino acids from cheap carbon and nitrogen sources. Also, fermentative methods seem to be the most economical and practicable means of producing lysine and many of such processes have been investigated (Schrumpf *et al.*, 1992; Eggeling, 1994; Ekwealor and Orafu, 2003).

Lysine is utilized in the pharmaceutical industry in the development of diet with balanced composition and in amino acid infusion (Costa-Ferreira and Duarte, 1992); in the animal feed industry for incorporation into the poultry and swine feed and in the cosmetic industry(Shah and Hameed,2004). Also a major segment of the population of Nigerians depend on cereal grains which lack some of the essential amino acids, including lysine. The amino acid supplementation of lysine in the diet will without exception increase the quality of cereal grains. The requirements of Nigerian industries for the amino acid is met only through importation, which involves spending huge amount of foreign exchange. However, lysine can be produced locally by microbiological methods using available raw materials such as millet, sorghum, seed meals. The bioconversion of these agricultural products into lysine may increase its economic importance. The present work is an attempt to develop a fermentation process that could produce lysine and also meet the economic needs of the country.

Materials and Methods

Microorganism used:

The culture of *Escherichia coli* NCCB 1841, a lysine-requiring auxotroph was purchased from the Netherlands Culture Collection of Bacteria. It was grown in a medium with the following composition (g/l): peptone, 10.0; yeast extract, 10.0; NaCl, 5.0 water, 1litre; pH7.2, incubation temperature 37^0C. Culture of the lysine auxotroph was maintained on Nutrient agar (Fluka) slants stored at 4^0C.

Collection of samples

Soil samples (34) from the rhizospheres of different plants of cassava, yam, and plantain including sandy and humus soils were collected from Awka town and used for this study. The soil samples were randomly collected from farmlands at a depth of 5-10cm. In each location, sterile polythene bags were used to collect the soil samples and then taken to the lab for analysis.

Isolation of Bacteria from Soil

One gram of soil sample was aseptically transferred into 9ml of sterile physiological saline in a test tube and shaken thoroughly for 2min.For heavy soil paricles, the suspension was allowed to stand for 1min.Thereafter, a tenfold serial dilution was done and aliquots of 0.1ml of 10^{-6} of dilution were used to inoculate plates of Nutrient agar (Fluka) and Tryptone Soy agar (Oxoid). The plates were incubated at 30^0C for 24h. Colonies that developed were subcultured and pure cultures preserved at 4^0C on sterile nutrient agar slants.

Screening of bacteria for lysine production on solid agar medium

The isolates were screened for lysine production using a minimal medium consisting of glucose, 4.0g; $(NH_4)_2SO4$, 2.0; K_2HPO_4, 0.5g; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.001g; $FeSO_4.7H_2O$, 0.0O1g; $MnSO_4.4H_20$, 0.00lg; $CaCO_3$, 2g; Agar, 15.0g, water, 1 litre of water with pH adjusted to 7.2. The medium which was seeded with a 24h broth culture of the lysine auxotroph, *Escherichia coli* (NCCB 1841), was inoculated with the isolate by spreading technique. An uninoculated agar plate served as control. After 72h incubation at 30^0C, the plates were examined for growth of the auxotroph. Halo growth of the *E.coli* indicates lysine production by the isolate. The lysine producers were stored for further studies. A total of 460 bacterial isolates were screened and 37 of them were selected as lysine producers.

Lysine production in submerged culture

Inoculum preparation

Two loopful of the isolate (24h) was grown in an Erlenmeyer flask containing 50ml of seed medium sterilized at 121^0C for 15min.The seed medium consists of peptone, 10.0g; yeast extract, 10.0g; NaCl, 5.0g; water, 1litre; pH adjusted to 7.2. The flask was incubated for 24h on a rotary shaker at 120rpm and 30^0C. Duplicate flasks were used

Fermentation

A cotton plugged Erlenmeyer flask(100ml) containing 20ml of fermentation medium: 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; $(NH_4)_2SO_4$, 10g; glucose, 20g; water, 1 litre; pH adjusted to 7.2, was used for lysine production. After sterilization the flask was cooled to room temperature and 1ml ($1.8x10^7$ cfu/ml) volume of the isolate was inoculated into the fermentation medium. Uninoculated flask served as control. The flask was incubated in a rotary shaker (160rpm) at 30^0C for 72h. Thereafter, lysine production, bacterial growth and residual sugar were determined from the broth culture.

Quantitative determination of lysine

Lysine in the broth culture was determined by acidic ninhydrin method of Chinard (1952). A 5ml volume of the culture broth of the isolate was centrifuged at 5000xg 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 100^0C 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.

Determination of growth of bacterial isolates

The growth of the bacterial isolates was determined turbidimetrically from the culture broth in Jenway (6405uv/vis) spectrophotometer at 660nm

Identification of bacterial isolates

The very active bacterial isolates were identified following

the methods described by Baron and Sydney (1990) and Holt *et al*., (1994).

Effect of medium volume on growth and lysine production

Various volumes (20–40ml) of fermentation medium consisting of: KH_2PO_4, 0.5g; K_2HPO_4, 0.5g; $MgSO_4.7H_2O$, 0.001g; $MnSO_4.H_2O$, 0.001g; $FeSO_4.7H_2O$, 0.001g; $CaCO_3$, 50g; glucose, 20g; $(NH_4)_2SO_4$, 10g; water, 1litre; pH adjusted to 7.2, were transferred to 100ml Erlenmeyer flasks. After sterilization the flasks were cooled to room temperature and inoculated with 1ml of a 24h seed inoculum of the *Bacillus* species. The flasks were placed on a rotary incubator shaker (160rpm) at 30^0C for 72h.An uninoculated flask was kept as control. Lysine concentration and bacterial growth were determined as previously described. The medium volume that gave the highest lysine concentration was used for further studies.

Effect of inoculum size

Various inoculum sizes(4-20%) were inoculated into Erlenmeyer flasks (100ml) containing 20ml of the medium composed of KH_2PO_4, 0.5g; K_2HPO_4, 0.5g; $MgSO_4.7H_2O$, 0.001g; $MnSO_4.H_2O$, 0.001g; $FeSO_4.7H_2O$, 0.001g; $CaCO_3$, 50g; glucose, 20g; $(NH_4)_2SO_4$, 10g; Water, 1litre; pH 7.2 for *Bacillus subtilis* PR13 *and Bacillus pumilus* SS16 and 25ml of a similar medium for *Bacillus subtilis* PR9.After sterilization the flasks were placed on a rotary shaker (160rpm) at 30^0C for 72h. An uninoculated flask was used as control. Lysine concentration and bacterial growth were determined as previously described. The inoculum size that gave the maximum lysine concentration was used for further studies.

Results

Isolation and screening for lysine production on solid agar medium

A total of 460 bacterial isolates were screened and 37 were recovered as active lysine producers. The bacterial isolates (37) secreted lysine in the agar medium which stimulated the growth of the lysine auxotroph, *Escherichia coli* seeded in the agar medium. The degree of the halo growth of the isolates is as shown in Table 1, with isolates HS26, PR13, SS67, PR29, PR9, HS60 and SS16 producing high lysine yields.

Lysine production in submerged culture

Table 2 shows the result of lysine production by the isolates in submerged culture. From the result (Table 3) isolates PR9, PR13 and SS16 are the most active lysine producers.

Identification of bacterial isolates

The morphological and biochemical characteristics of the three active lysine producers are presented in Table 3. All the isolates showed good growth on Nutrient agar. The isolates were Gram positive rods, endospore formers and they have milky white colonies. They hydrolyzed casein, gelatine and starch except isolate SS16 which did not hydrolyze starch.Based on the characteristics features of the isolates they were identified as *Bacillus subtilis* PR13, *Bacillus subtilis* PR 9 *and Bacillus pumilus* SS16.

Effect of medium volume on growth and lysine production

The results of the effect of medium volume on growth and lysine production by *Bacillus subtilis* PR13, *Bacillus subtilis* PR 9 *and Bacillus pumilus* SS16 are presented in Fig 1-3.The results show that maximum lysine yields were stimulated by Bacillus *subtilis* PR13 and Bacillus *subtilis* PR9 in a medium volume of 20ml (fig 1and 3), while Bacillus *pumilus* SS16 accumulated the highest lysine yield in a medium volume of 25ml (Fig 2). The highest lysine concentration of 1.88mg/ml was produced by *Bacillus subtilis* PR13.

Effect of inoculum size

The results of the effect of inoculum size on growth and lysine production *Bacillus subtilis* PR13, *Bacillus subtilis* PR 9 *and Bacillus pumilus* SS16 are shown in Fig 4-6. The results show that maximum lysine yields were achieved by *Bacillus subtilis* PR13 *and Bacillus pumilus* SS16 at 10% inoculum size(Fig 4 and 6), while *Bacillus subtilis* PR9 accumulated the highest lysine yield of 8% inoculum size. The highest lysine yield of 1.94mg/ml was obtained by *Bacillus subtilis* PR13.

Table 1: Screening for lysine production on solid medium

Bacterial isolate No./code	Degree of halo growth of *Escherichia coli*
CR 58	++
HS 26	++++
HS 37	++
HS 8	++
PR 71	++
PR 13	++++
SS 67	++++
YR 54	++
YR 45	+++
YR 4	++
YR 20	+++
PR 29	++++
SS 32	++
SS 44	++
SS 6	+++
PR 4	++
PR 42	+++
PR 67	++
PR 34	++
PR 9	++++
PR 25	++
PR 56	++
PR 2	+++
HS 60	++++
HS 52	++
HS 13	+++
HS 40	++
SS 38	+++
SS 20	+
SS 16	++++
YR 13	++
YR 7	+
YR 3	+++
YR 36	++
CR 65	+++
CR 10	++
CR 24	+++

Result interpretation: + very low lysine producers; ++ low lysine producers; +++ moderate lysine producers; ++++ high lysine producers

Table 3: Production of lysine in submerged medium

Bacterial Isolate No./code	Lysine (mg/ml)
CR 58	0.61
HS 26	1.20
HS 37	0.64
HS 8	0.45
PR 71	0.26
PR 13	1.45
SS 67	1.14
YR 54	0.43
YR 45	0.92
YR 4	0.55
YR 20	0.92
PR 29	1.16
SS 32	0.20
SS 44	0.36
SS 6	0.91
PR 4	0.23
PR 42	0.98
PR 67	0.37
PR 34	0.25
PR 9	1.54
PR 25	0.58
PR 56	0.27
PR 2	0.81
HS 60	1.17
HS 52	0.36
HS 13	0.94
HS 40	0.37
SS 38	0.87
SS 20	0.30
SS 16	1.35
YR 13	0.42
YR 7	0.27
YR 3	0.96
YR 36	0.60
CR 65	1.05
CR 10	0.46
CR 24	1.01

Table 3: Morphological and Biochemical characteristics of Bacteral Isolates.

Code	Colonial Morphology	Gram reaction	Catalase	Spore test	Motility	Oxidase test	Urease test	Methyl red test	Voges Proskauer	Citrate test	Starch hydrolysis	Casein hydrolysis	Gelatin hydrolysis	Indole test	Growth at 45 ^{0}C	Growth at 65^{0}C	Growth at 7% NaCl	Glucose	Arabinose	Lactose	Mannitol	Probable identity
PR 13	Milky white colonies	+ rods	+	+	+	+	-	-	-	+	+	+	+	-	+	-	+	+	+	+	+	*Bacillus subtilis* PR 13
PR 9	Milky white colonies	+ rods	+	+	+	+	-	-	-	+	+	+	+	-	+	-	+	+	+	+	+	*Bacillus subtilis* PR 9
SS 16	Milky white colonies	+ rods	+	+	+	+	-	-	+	+	-	+	+	-	+	-	+	+	+	+	+	*Bacillus pumilus* SS 16

Key:
+ = positive
- = negative

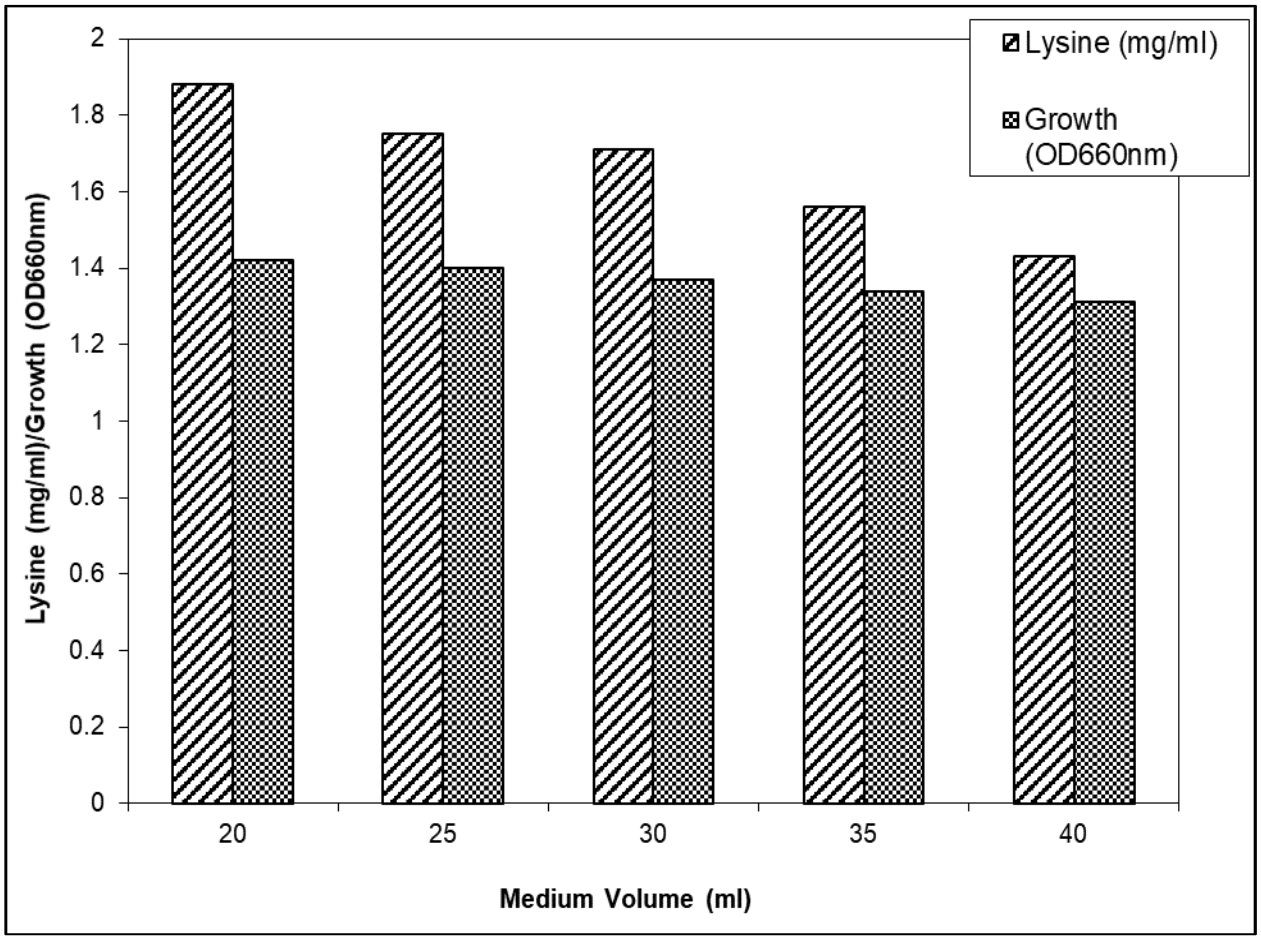

Fig 1: Effect of Medium Volume on Lysine Production by *Bacillus subtilis* PR13

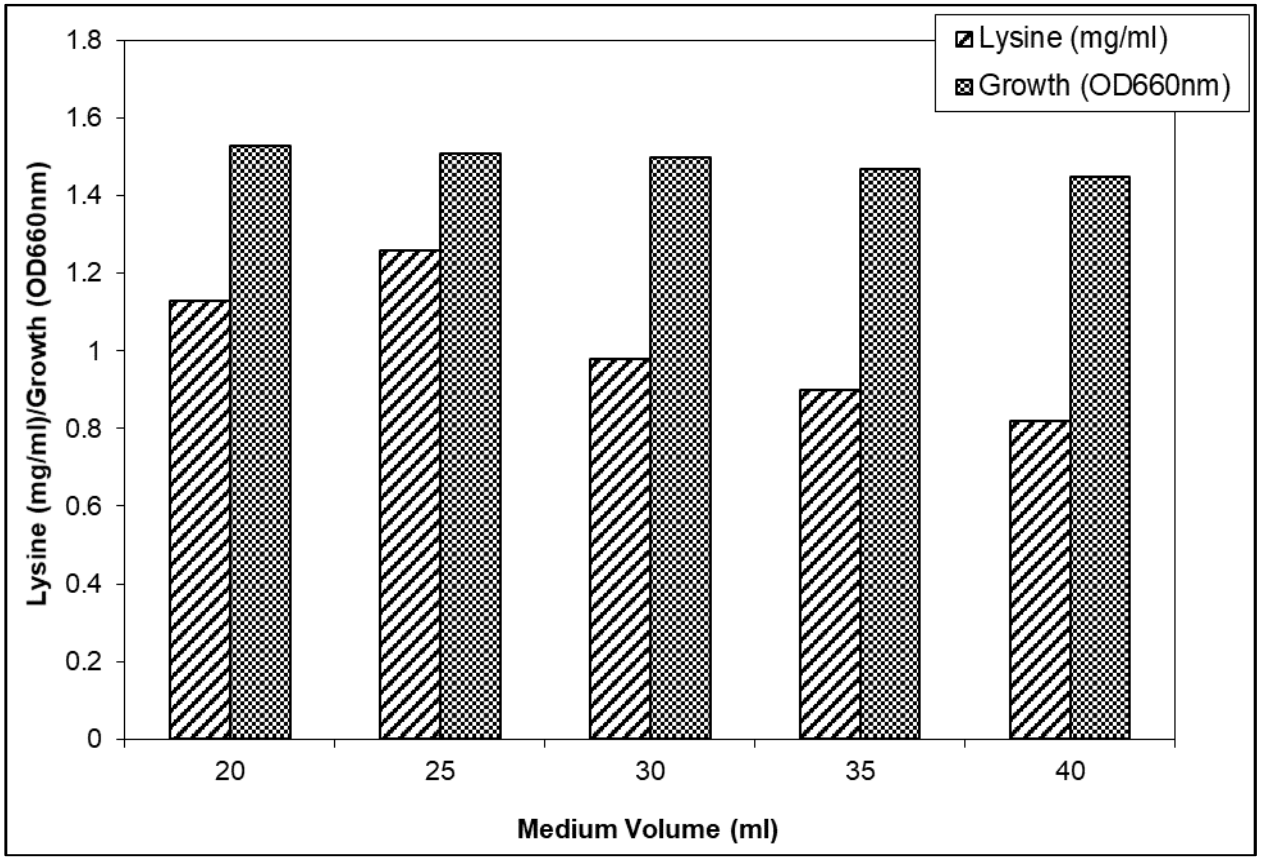

Fig 2: Effect of Medium Volume on Lysine Production by *Bacillus subtilis* PR9

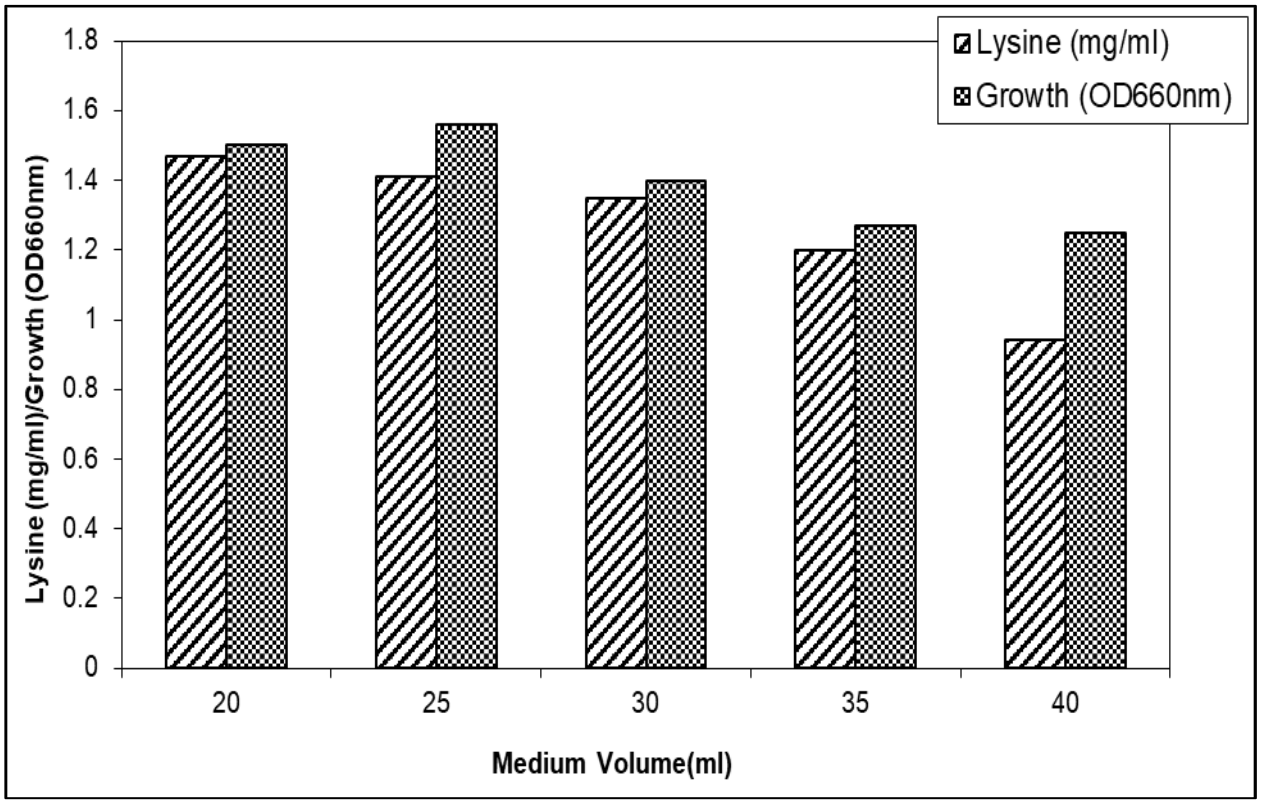

Fig 3: Effect of Medium Volume on Lysine Production by *Bacillus pumilus* SS16

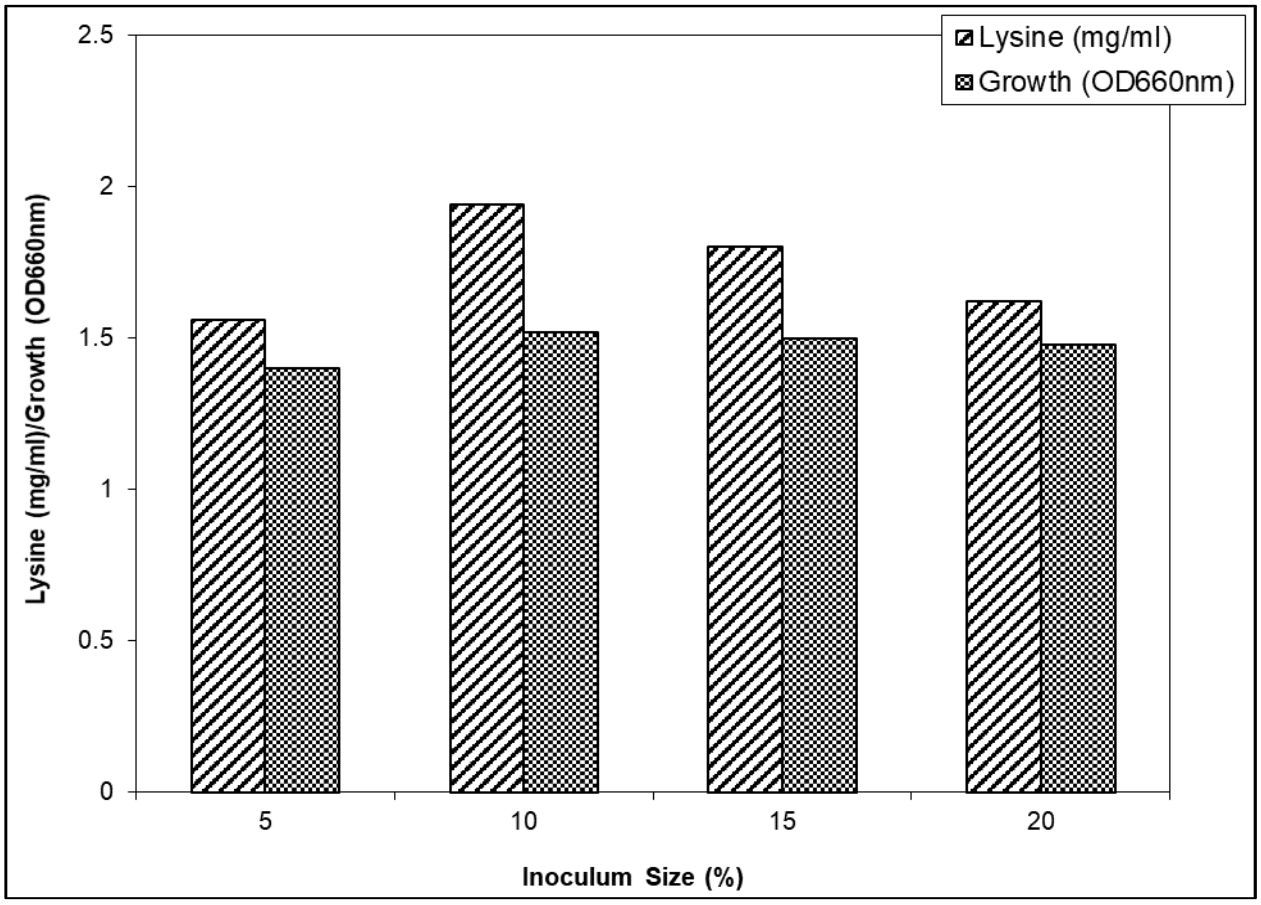

Fig 4: Effect of Inoculum Size on Lysine Production by *Bacillus subtills* PR13

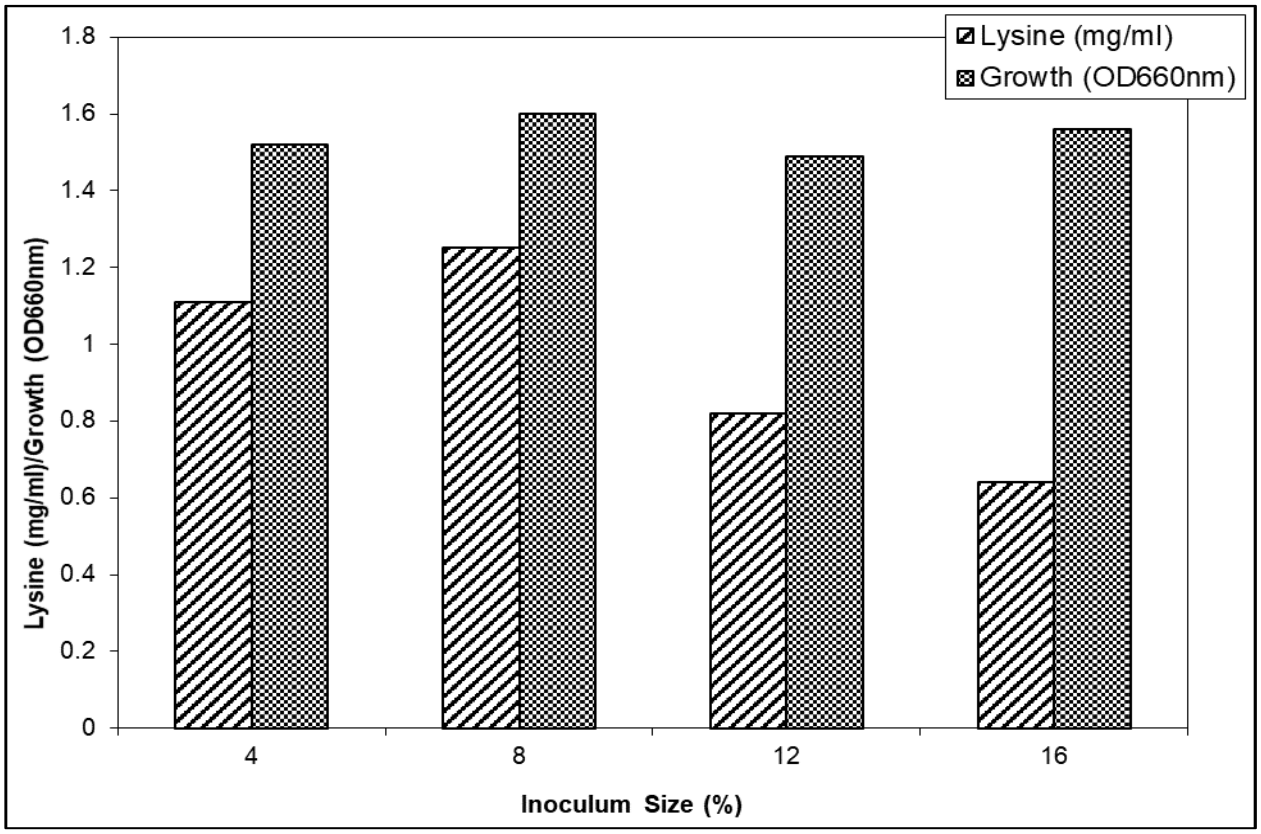

Fig 5: Effect of Inoculum Size on Lysine Production by *Bacillus subtilis PR9*

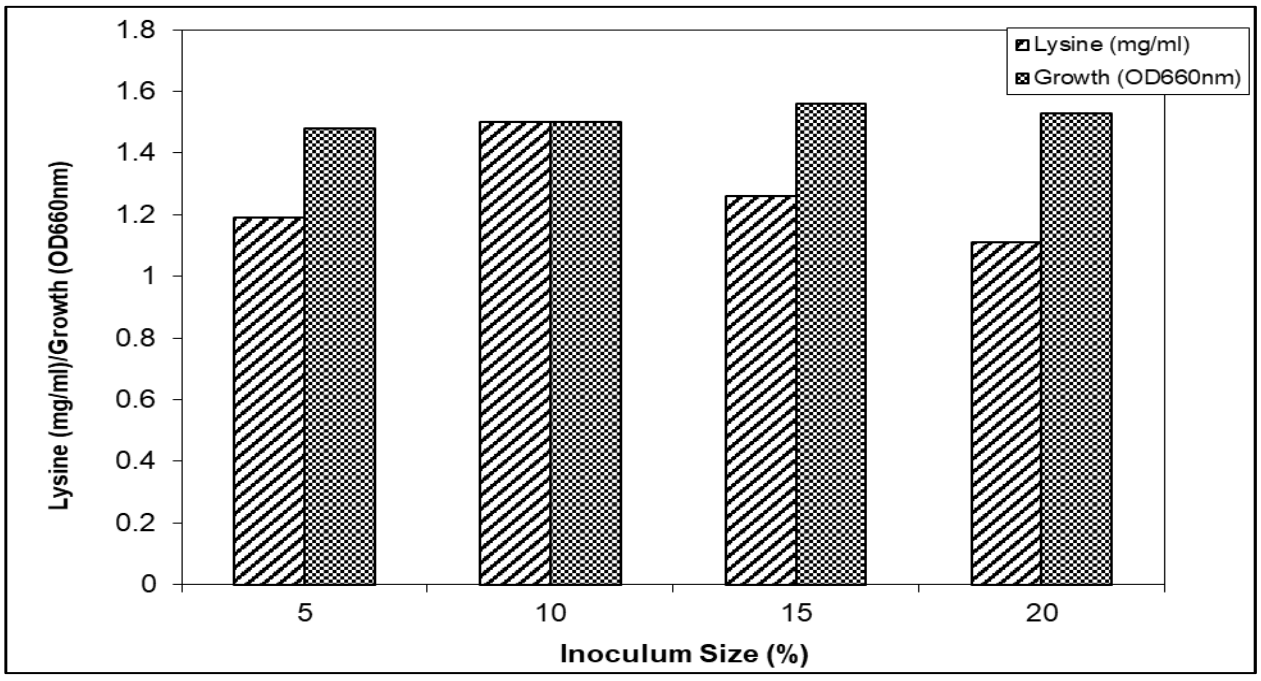

Fig 6: Effect of Inoculum Size on Lysine Production by *Bacillus pumilus* SS16

Discussion

The occurrence of lysine-producing organism from the soil agrees with earlier reports of Sen and Chatterjee, (1983) who isolated *Arthrobacter globiformis* from Burdwan (India) soil and found it able to accumulate 3.4g/l L-lysine in the growth medium. Also Ekwealor and Obeta (2005), recovered a lysine-producing bacterium *Bacillus megaterium* sp14 from the soil and was found to accumulate a lysine yield of 3.56 mg/ml in the broth culture. Sen *et al* (1983) isolated *Micrococcus varians* from Assam (India) soil and was able to accumulate 2.6g/l L-lysine in a purely synthetic medium under optimal conditions. Siripoke *et al.* (1998) and Umerie *et al.* (2000) also reported the isolation of high lysine producing *Bacillus* species form the local soil.

The bacterial isolates that were observed to produce the highest amount of lysine were identified as *Bacillus* species. Similar observations have also been reported by Siripoke *et al* (2005) and Ekwealor and Obeta (2005), who selected *Bacillus* SWU41 and *Bacillus megaterium* SP14 as the highest lysine producers respectively.

In the study the optimum medium volume were 20ml for

B.subtilis PR13 and *B.pumilus SS16* and 25ml for *B.subtilis* PR9. The recording of best volume of 25ml by *B.subtilis* PR9 was in line with the report of Ekwealor and Obeta (2005), who observed that lysine production by *Bacillus megaterium* SP14 increased with increasing medium volume ratio up to 25%.Further increase caused a decrease in lysine synthesis. Other workers presented a contradictory report, Shah *et al.* (2002) reported maximum lysine production of 15.05g/l and sugar utilization of 6.7% when 50ml broth volume was utilized. As the volume increased, L-lysine production and sugar utilization decreased, as observed with 150ml culture volume only 8.55g/l L-lysine was produced and 4.85% sugar utilized. Dike and Ekwealor (2012), reported maximum growth and methionine production in flask containing 30ml broth volume for *Bacillus cereus* DS13 and *B. cereus* RS16 and 40ml for *B.* cereus AS9. Ganguly and Banik (2010), also reported maximum L-glutamic acid production by mutant of *Micrococcus glutamicus* in the flask containing 30ml of fermentation broth. Inbar *et al.* (1985), reported the appearance of lactate and succinate at low aeration that was attributed to the increased glycolysis. Oxygen an importat nutrient is usually supplied to flask through vigorous shaking on a rotary or reciprocal shaker. Under condition of insufficient oxygen large amount of lactic and succinic acids were accumulated, while excess of oxygen increase the of α-ketoglutaric acid (Shah *et al*., 2002). It was found that both over abundance and meager aeration were undesirable. The former being inhibitory to cell growth and the latter to L-lysine production (Hallaert *et al*., 1987; Inbar *et al*., 1985; Liu, 1986). Hadj-Sassi *et al.,* (1996) studied the effect of O_2, on lysine production, biomass formation and substrate consumption for *Corynebacterium glutamicum* ATTCC 21513 and found that O2 limitation caused a decrease in substrate consumption rate and conversion efficiency of substrate into L-lysine. The maximum conversion rate into L-lysine was obtained at 30-35% without CO_2 addition and redox potential of 440mv.

The production of lysine was increased with increase in the size of inoculum which was found to be optimal at 8% for *Bacillus pumilus* SS16 and 10% for *B.subtilis* PR13 and *B.subtilis* PR9.This finding is in line with report of other workers. Ekwealor and Obeta (2005) observed maximum L-lysine production by *Bacillus megaterium* SP14 using 10% inoculum size. Dike and Ekwealor (2012), similarly reported maximum growth and methionine production by *B. cereus* strains using 10% inoculum size. Nakayama *et al.* (1961), utilized 10% inoculum size for various auxotrophic mutants. It may not be fair to say that 10% inoculum size is required in all cases of fermentation, since it depends upon the cell mass and the composition of the seed medium to be transferred. Shah *et al.* (2002), reported that maximum lysine production (15.3g/l) for *Corynebacterium glutamicum* was obtained at 10% inoculum size. Hallaert *et al.,* (1987) reported that inoculum size has a marked effect in fermentation. He opined that small inoculum size causes an increase in growth period because a very low inoculum density may give insufficient biomass. Other workers presented contradictory reports, Avendano and Cornejo (1987), Acharya and Chaudhary (2012) and Ray *et al*. (2007) found 2% inoculum size to be better for maximum enzyme production. Adnan *et al.* (2011), observed maximum L-lysine (23.5735g/kg) production in *Brevibacterium linens* DSM 20158 when 4.0% inoculum size was used, thereafter there was a decline in the yield. Vegetative growth may be promoted at the cost of L-lysine production due to competition for available nutrients (Reddy *et al*., 2008).It is generally necessary to optimize inoculum density. Too low a density may give insufficient biomass that cannot deplete the substrate of nutrients necessary for L-lysine fermentation.

As the inoculum level was further increased in the study, the production of the lysine gradually reduced. It is likely that at a higher inoculum level, the bacteria grow rapidly and the nutrient essential for the growth of bacteria were consumed at the initial stages that resulted in the accumulation of other by products in the fermentation medium. Also it was suggested that higher inoculum size resulted in reduced dissolved oxygen and increased competition towards nutrient (Rahman *et al*., 2005). The insignificance of the results at low levels of the inoculums may have been due to the slow growth of the organism and increase in the time period for the bacteria to reach the stationary phase. Shah *et al.* (2002), opined that high density may produce too much biomass and deplete the substrate of nutrients necessary for product formation.

In conclusion, the study has clearly revealed that lysine producing *Bacillus* species can be isolated from the soil

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