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Studies on Lysine Accumulation in the Broth Culture of *Bacillus* Species using Carbohydrates as Carbon Sources and Seed Meals as Nitrogen Sources

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ABSTRACT
Lysine production in the broth culture of *Bacillus* species using carbohydrates as carbon source and seed meals as nitrogen source was investigated. Different carbohydrate and proteins seeds were sourced from an open market in Awka Anambra State South Eastern Nigeria and prepared in the laboratory using standard procedures. The carbohydrates(carbon source) and seed meals(nitrogen source) were added into Erlenmeyer flasks containing the basal medium and inoculated with different cultures of *Bacillus subtilis* PR13, *B. subtilis* PR9 and *B. pumilus* SS16. Maize hydrolysate recorded the highest reducing sugar(5.2mg/ml), followed by sorghum(4.8mg/ml) and the least was recorded by sweet potato(2.1mg/ml).The best carbon source for maximum lysine yield by *B. subtilis* PR13 was millet, while for *B. subtilis* PR9 and *B. pumilus* SS16 it was sorghum respectively. Maximum lysine production by *B. subtilis* PR13 was stimulated at a millet concentration of 6%, while enhanced lysine yield by *B. subtilis* PR9 and *B. pumilus* SS16 was observed at a sorghum concentration of 6%. The best nitrogen source for enhanced lysine yield by *B. subtilis* PR13 and *B. pumilus* SS16 was soyabean meal respectively, while for *B. subtilis* PR9 the best was peanut meal. Optimum lysine yield by *B. subtilis* PR13 and *B. pumilus* SS16 was observed at soyabean concentrations of 4% and 2% respectively, while maximum lysine accumulation by *B. subtilis* PR9 was observed at 4%. These findings indicate appreciable lysine production capability of *Bacillus* species when agricultural products are used as carbon and nitrogen sources.

Keywords: *Lysine production, broth, Bacillus species, carbohydrates, seed meals*

INTRODUCTION
Out of the twenty naturally occurring amino acids, L-lysine is one of the nine essential amino acids and commercially important amino acids. Lysine is an essential amino acid required mainly by children and growing animals. It cannot be synthesized biologically in the body (Shah *et al.,* 2002), but may be added to food and feed materials to improve the protein quality (Stilling *et al.,* 1971). Of that manufactured commercially, the largest amount 80% is produced by fermentation and 20% by chemical synthesis (Coello, *et al.,* 2001). L-lysine is presently been used in the pharmaceutical, food, feed milling and cosmetics industries. Thus, the outlook for this amino acid is high because of its expanding market demand (Anastassiadis, 2007). The main difference between L-lysine produced for human consumption and that produced for animal feed is the level of purification of the final product. For human consumption, the form is typically that of a fine chemical used as part of a supplement or higher chemical application; In contrast, lysine for animal feed can be within the purity range of 35 to 80% (Anastassiadis, 2007). To determine the purity of the final product, downstream processing options including ion exchange chromatography and drying can be chosen.

L-lysine was discovered to be produced through fermentation by *Corynebacterium glutamicum* at Kyowa Hakko's plant in 1958 in Japan (Kelle *et al*., 2005). This method is preferred over all other methods because it employs low temperature, low pressure, low cost carbon sources and renders biological form of L-lysine as the final product (Nasab *et al*., 2007). Microorganisms that have been reported to produce L-lysine include *Corynebacterium glutamicum* (Nelofer *et al*., 2008), *Bacillus megaterium* (Ekwealor and Obeta, 2005), *Brevibacterium linens*, *Streptomyces Albulus IFO* (Shih *et al*., 2006), *Brevibacterium flavum*, *M. methylophilis* (Ishikawa *et al*., 2008), *B. lactofermentum* (Tosaka *et al*., 1979), *B. subtilis* and *Bacillus laterosporus* (Umerie *et al*., 2000). Among these, *C. glutamicum* has been most widely exploited industrially for L-lysine production (Pfefferle *et al*., 2003). There are other different ways of L-lysine production which include chemical synthesis, enzymatic method, extraction from protein hydrolysate, recombinant DNA technology and protoplast fusion (Anastassiadis, 2007; Nelofer *et al.,* 2008).

Agro-industrial by – products are being used as nitrogen and carbon source in lysine production(Ekwealor and Ebele , 2003).Sugar cane molasses is a cheap carbon source, containing sucrose, glucose and fructose at a total carbohydrate content of 50 to 60%(Reed, 1982). Glucose, fructose and sucrose are important carbon sources that have a pronounced effect on kinetics and stoichiometry of lysine production by *C. glutamicum*(Kiefer *et al.,*2002). It provides a source of fermentable sugars as well as some elemental nutrients, which plays key role in the fermentation process.

Nutritional and physical parameters affect the growth and product yield of organism (Wang *et al*., 1991; Coello *et al*., 1992). 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. Product yield of organism is also strongly affected by the rate of sugar utilization or growth rate of a strain.

The aim of the research was to study the influence of different carbohydrates and seed meals on lysine production.

MATERIALS AND METHODS

Collection of Samples

Different carbohydrate samples such as cassava (*Mannihot utilissima*), yam (*Dioscorea rotundata*), millet (*Bajra pennisetum americanum*), sweet potato (*Ipomea batatas*), sorghum (*Sorghum bicolor*), maize (*Zea mays*) and rice (*Oryza sativa*) were purchased at the local market in Awka, Anambra State.

Preparation of carbon sources

They were prepared into starches in the laboratory according to standard procedures as described by Odibo (1987). Starch substrates were prepared from tubers (cassava, yam, sweet potato) and grains (rice, corn sorghum, millet). The tubers were cleaned to remove soil particles, peeled and washed (thrice) in clean water. They were cut into small pieces before being homogenized with water in a blender. Grains were soaked for 48h to soften the seeds and then homogenized with water. The resulting mash or homogenate was collected in a white muslin cloth, squeezed to extract the starch (in suspension) into plastic bowl and allowed to settle. The supernatant was decanted and the sedimented starch dried at 50^0C for 48h. The resultant flakes were ground into fine powder and used as native starch.

Enzyme hydrolysis of carbon sources for reducing sugar production

The enzyme hydrolysis was done following the method described by Umerie *et al.* (2000).A 500ml beaker containing 30g of the starch in 100ml of water was heated for 15min at 100^0C to gelatinize the starch. The beaker was covered with an aluminium foil after adding 1.0ml of α-amylase and heated again in a water bath for 10min at 95^0C to effect liquefaction. After cooling the liquefied starch to 60^0C, 1.0ml amyloglucosidase was added before replacing the beaker in the bath at 60^0C for 48h, for saccharification to take place.

Preparation of nitrogen sources

The protein seeds used cowpea (*Vigna unguiculata*), soyabeans (*Glycine max*), groundnuts(*Arachis hypogea*), bambara nuts (*Vignia subterranean*), were ground using a blender(Sonik, Japan). The seed meal (20.0g) in 200ml solvent(chloroform-ethanol mixture 2:1) was defatted by the soxhlet extraction method. After extraction for 6h, the defatted meal was then oven-dried at 50^0C for 20h.

Lysine production in submerged culture

Inoculum preparation

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

Effect of carbon sources

The effect of carbon sources (cassava, yam, sweet potato, millet, sorghum, maize, and rice) on growth and lysine production was examined. Various carbon sources(20g) were added 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; $(NH_4)_2SO_4$, 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 inoculated with 2ml of a 24h seed inoculum of the *Bacillus* species and placed on a rotary shaker (160rpm) at 30^0C for 72h. Thereafter, lysine production, bacterial growth and residual sugar were determined from the broth culture. The carbon source that gave the maximum lysine production was used for further studies.

Effect of concentrations of carbon sources

The effect of different concentrations (20-100g/l) of millet for *B. subtilis* PR13 and sorghum for *B. subtilis* PR9 and *B. pumilus* SS16 on growth and lysine production was determined. The fermentation process was carried out as previously described, and residual sugar, bacterial growth and lysine production determined after 72h.

Effect of nitrogen sources

Various nitrogen sources: peanut meal, bambara nut meal, soyabean meal, cowpea meal and their defatted seed meals were investigated for their effects on growth and lysine production. Various nitrogen sources(10g) were added 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; Water, 1litre; pH 7.2 for *B. subtilis* PR13 *and B. pumilus* SS16 and 25ml of a similar medium for *B. subtilis* PR9.The carbon sources of the fermentation medium were millet 60.0g for *B. subtilis* PR13 and sorghum 60.0g for both *B. subtilis* PR9 and *B. pumilus* SS16. After sterilization the flasks were inoculated with 2ml of a 24h seed inoculum of the *Bacillus* species and placed on a rotary shaker (160rpm) at 30^0C for 72h. Residual sugar, bacterial growth and lysine production were determined as previously described. The nitrogen source that gave the maximum lysine production was used for further studies.

Effect of varying concentrations of nitrogen sources

The effects of varying concentrations (10-80g/l) of soyabean meal on growth and lysine production by *B. subtilis* PR13, defatted peanut meal for *B. subtilis* PR9 and deffated soya bean meal for *B. pumilus* SS16 were investigated. Fermentation process was carried out as previously described, and residual sugar, bacterial growth and lysine production determined after 72h.

Estimation of reducing sugar

The reducing sugar content of the saccharified starch was determined by dinitrosalicyclic acid (DNS) method of Miller (1959). The DNS reagent was prepared by adding 1.0g of dinitrosalicyclic acid to 20ml of 2M NaOH and 30ml of distilled water. Potassium sodium tatarate (30g) was added and the volume of the mixture was increased to 100ml by the addition of distilled water. Reducing sugar was estimated by adding 1ml of DNS to 1ml of the hydrolyzed starch. The mixture was heated in a water bath at 100^0C 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 hydrolyzed sample with water. The reducing sugar content was subsequently determined by making reference to a standard curve of known glucose concentrations.

Quantitative determination of lysine

Lysine in the broth culture was determined by acidic ninhydrin method of Chinard(1952).

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

RESULTS

Enzyme hydrolysis of carbon sources for reducing sugar production

The results of enzyme hydrolysis of carbon sources for reducing sugar production are shown in Fig 1. Maize, sorghum and millet recorded 5.2mg/ml, 4.8 mg/ml and 4.4 mg/ml of reducing sugars respectively. Others were yam starch (3.9 mg/ml), rice starch (2.8 mg/ml), cassava starch (2.5 mg/ml) and sweet potato starch (2.1mg/ml).

Effect of carbon sources

The results of effect of carbon sources on growth and lysine production by *B. subtilis* PR13, *B. subtilis* PR 9 *and B. pumilus* SS16 are presented in Figs 2-4. The best carbon source for maximum lysine yield by *B. subtilis* PR13 was millet(Fig 2), while for *B. subtilis* PR9 and *B. pumilus* SS16 it was sorghum(Fig 3 and 4). *B. subtilis* PR13 produced the highest lysine yield of 1.71mg/ml and the residual sugar was 0.47mg/ml.

Effect of varying concentrations of carbon sources

The results of the effect of varying concentrations of carbon sources on growth and lysine production by *B. subtilis* PR13, *B. subtilis* PR 9 *and B. pumilus* SS16 are shown in Fig 11-13.The results show that maximum growth and lysine yields were observed in *B. subtilis* PR9 and *B. pumilus* SS16 when 60g/l sorghum was utilized (Fig 6 and 7), while *B. subtilis* PR13 accumulated the highest yield when 60g/l millet was utilized (Fig 5). The highest lysine yield of 2.08mg/ml was produced by *B. subtilis* PR13.

Effect of nitrogen sources

Figs 8-10 show the effect of nitrogen sources on growth and lysine production by *B. subtilis* PR13, *B. subtilis* PR 9 *and B. pumilus* SS16. The best nitrogen source for maximum lysine yield for *B. subtilis* PR 13 and *B.pumilus* SS16 was soyabean meal (Fig8 and fig 10), while for *B. subtilis* PR9 it was defatted peanut. *B. subtilis* PR13 produced the highest lysine yield of 2.21mg/ml and the residual sugar was 0.42mg/ml.

Effect of varying concentrations of nitrogen sources

The results of the effect of varying concentrations of nitrogen sources on growth and lysine production by *B. subtilis* PR13, *B. subtilis* PR 9 and *B. pumilus* SS16 are presented in Figs 11-13.The results show that maximum growth and lysine yields were observed in *B. subtilis* PR13 and *B. pumilus* SS16 when 40g/l and 20g/l of soyabean were utilized respectively(Fig11 and 12), while *B. subtilis* PR9 accumulated the highest yield when 40g/l of defatted peanut was used(Fig 13).

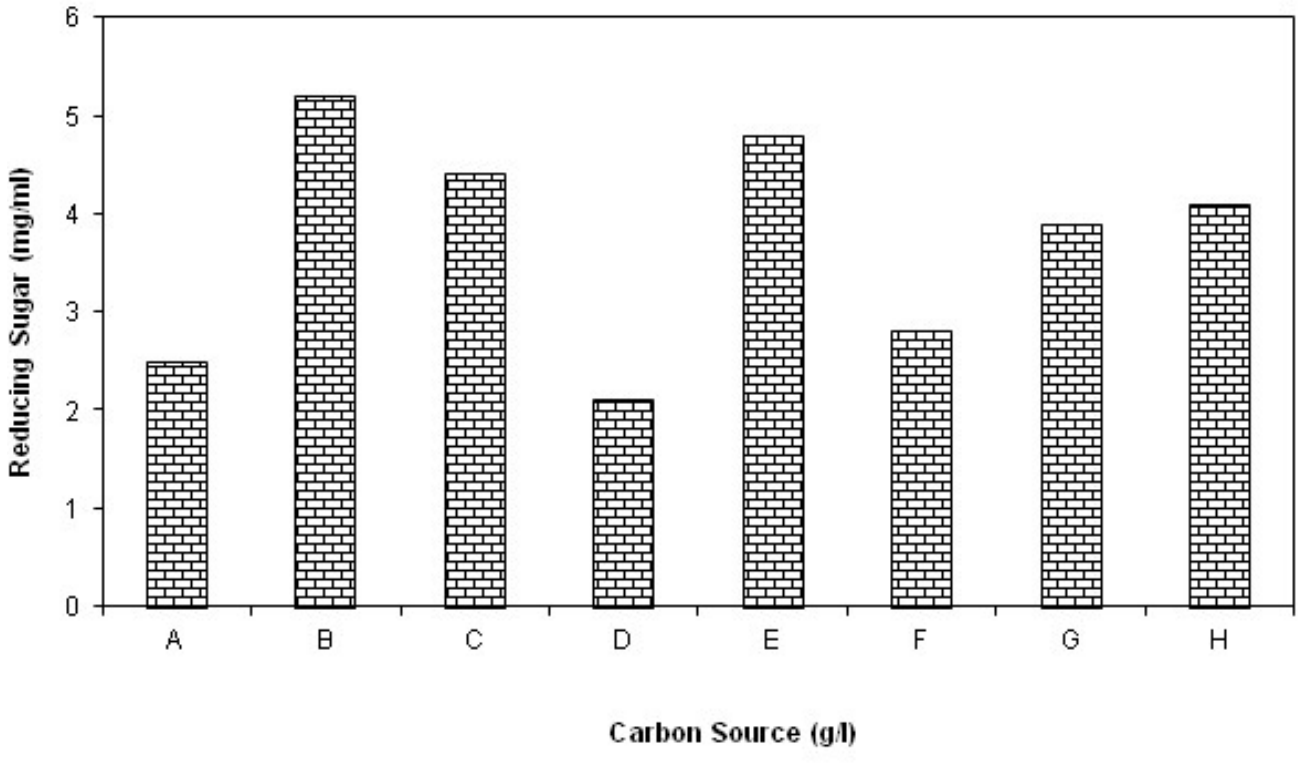

Figure 1: Enzyme Hydrolysis of Carbon Sources for Reducing Sugar Production A, Cassava; B, Maize; C, Millet; D, Sweet Potato; E, Sorghum; F, Rice; G, Yam; H, Commercial Starch

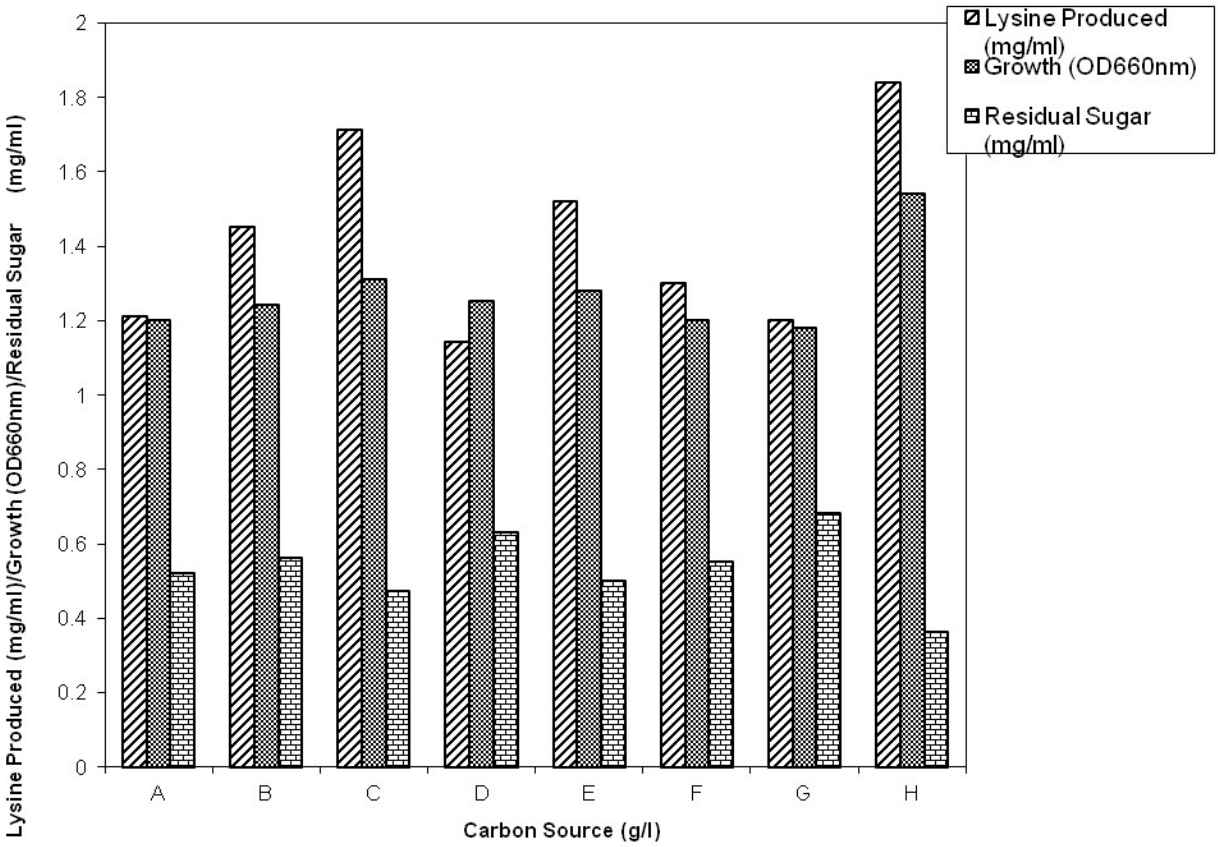

Figure 2: Effect of Carbon Sources on Lysine production by *Bacillus subtilis* PR13 A, Cassava; B, Maize; C, Millet; D, Sweet potato; E, Sorghum; F, Rice; G, Yam; H, glucose

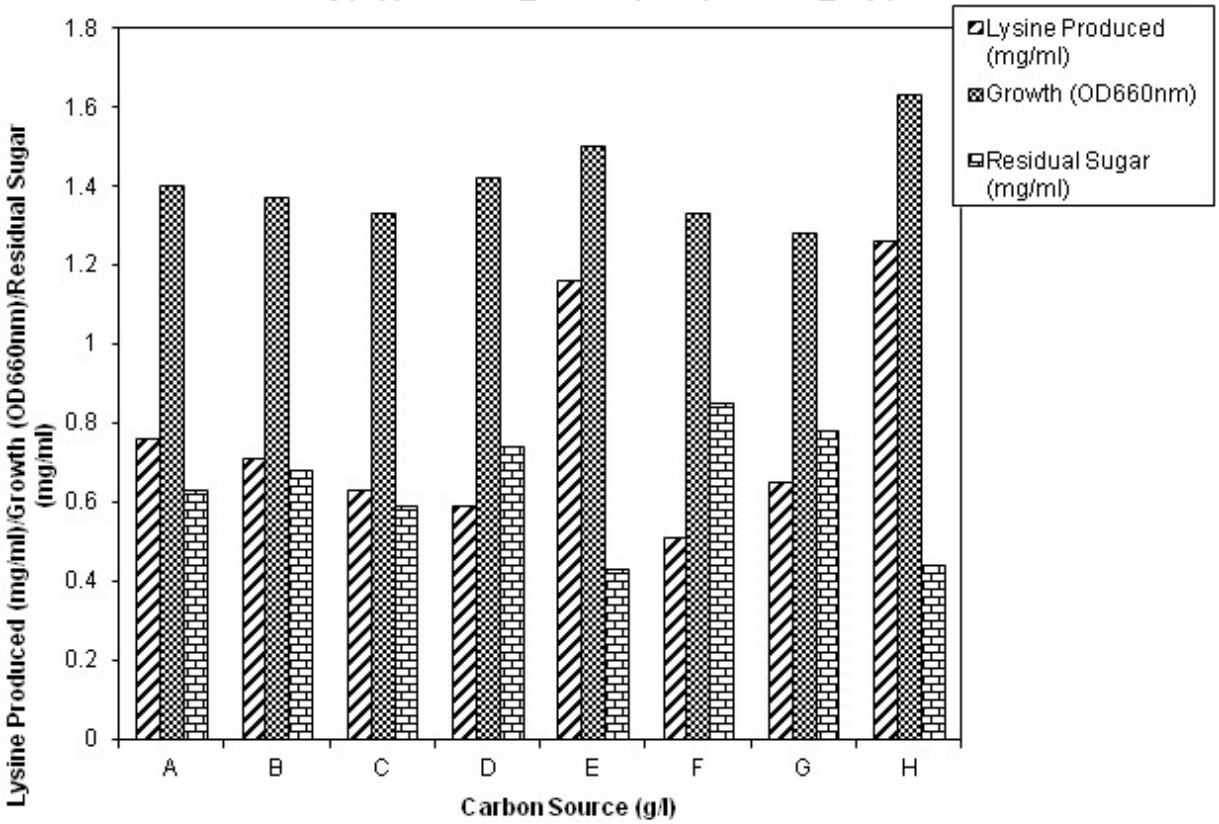

Figure 3: Effect of Carbon Sources on Lysine Production by *Bacillus subtilis* PR9: A, Cassava; B, Maize; C, Millet; D, Sweet Potato; E, Sorghum; F, Rice; G, Yam; H, glucose

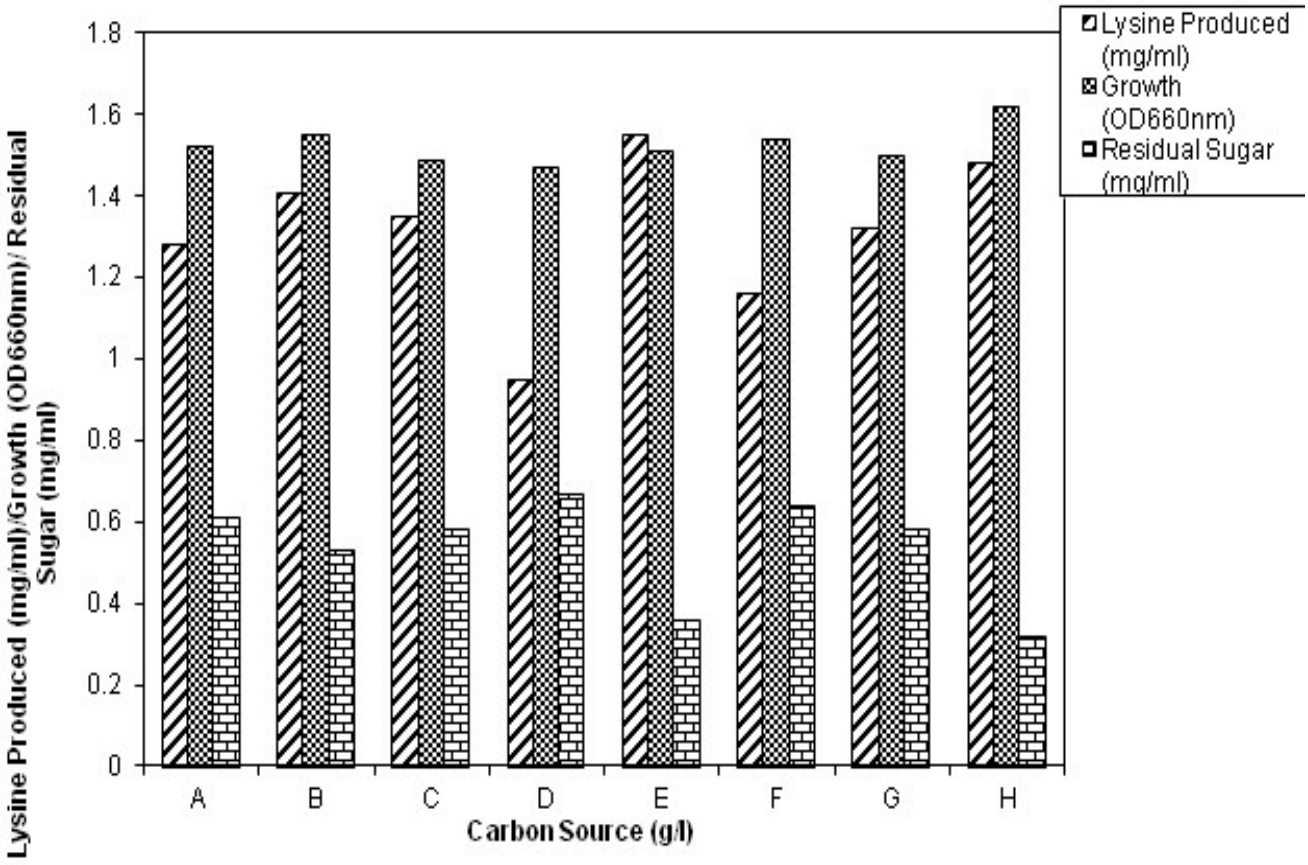

Figure 4: Effect of Carbon Sources on Lysine Production by *Bacillus pumilus* SS16 A, Cassava; B, Maize; C, Millet; D, Sweet Potato; E, Sorghum; F, Rice; G, Yam; H, glucose

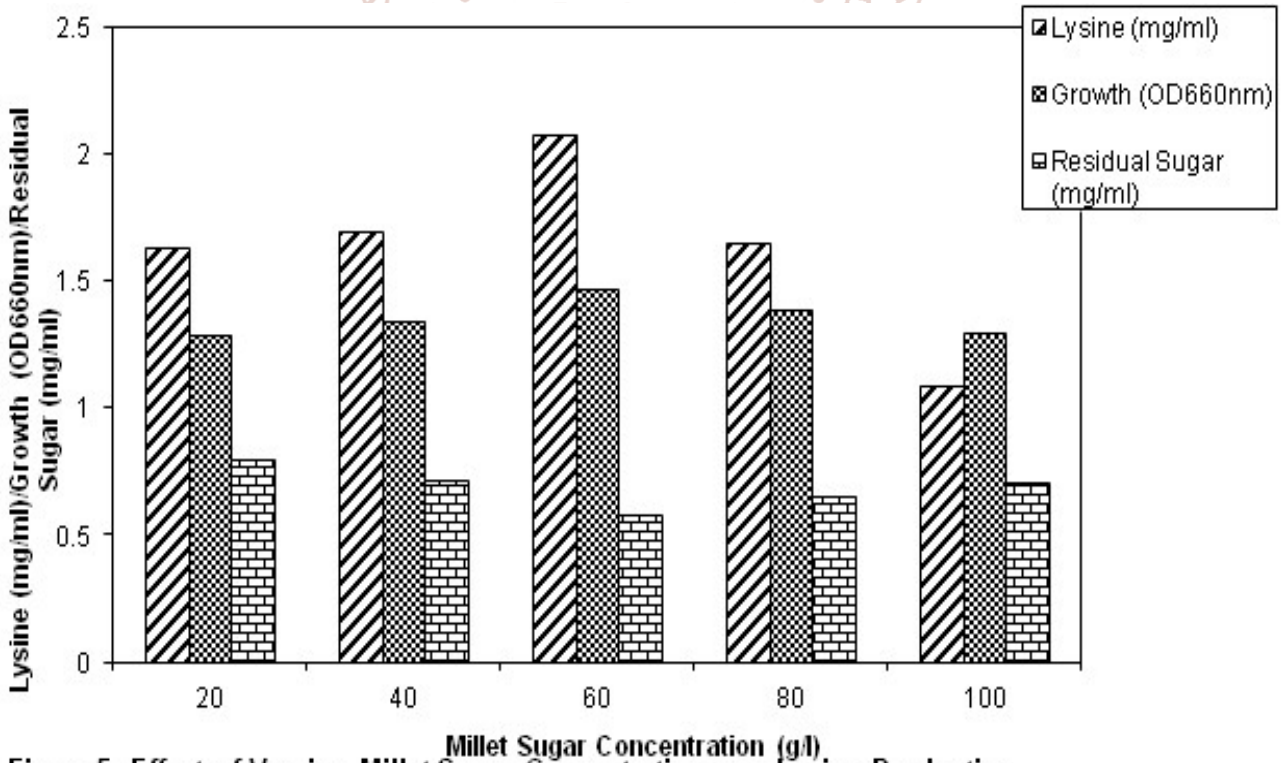

Figure 5: Effect of Varying Millet Sugar Concentrations on Lysine Production by *Bacillus subtilis* PR13

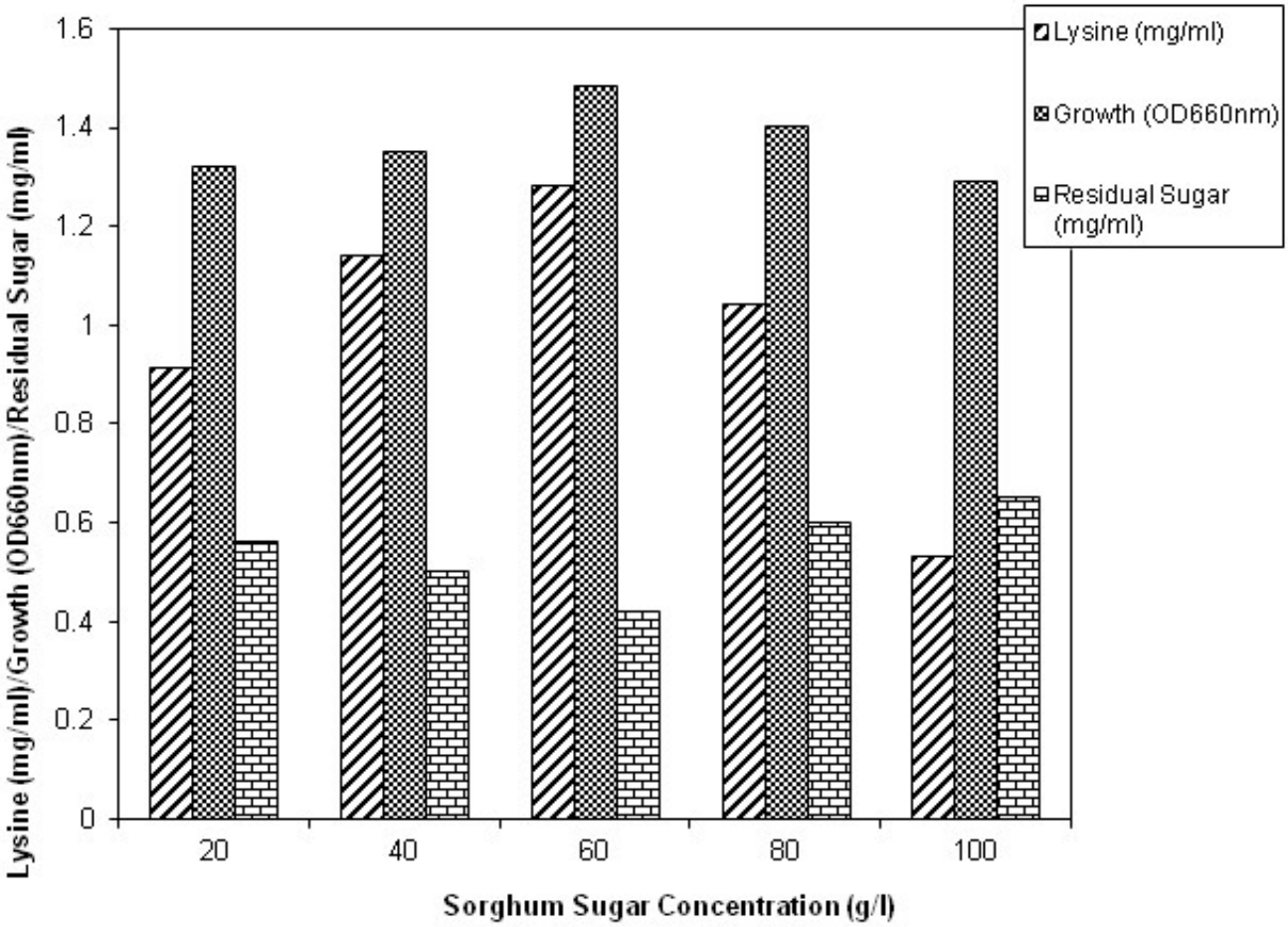

Figure 6: Effect of Varying Sorghum Sugar Concentrations on Lysine Production by *Bacillus subtilis* PR9

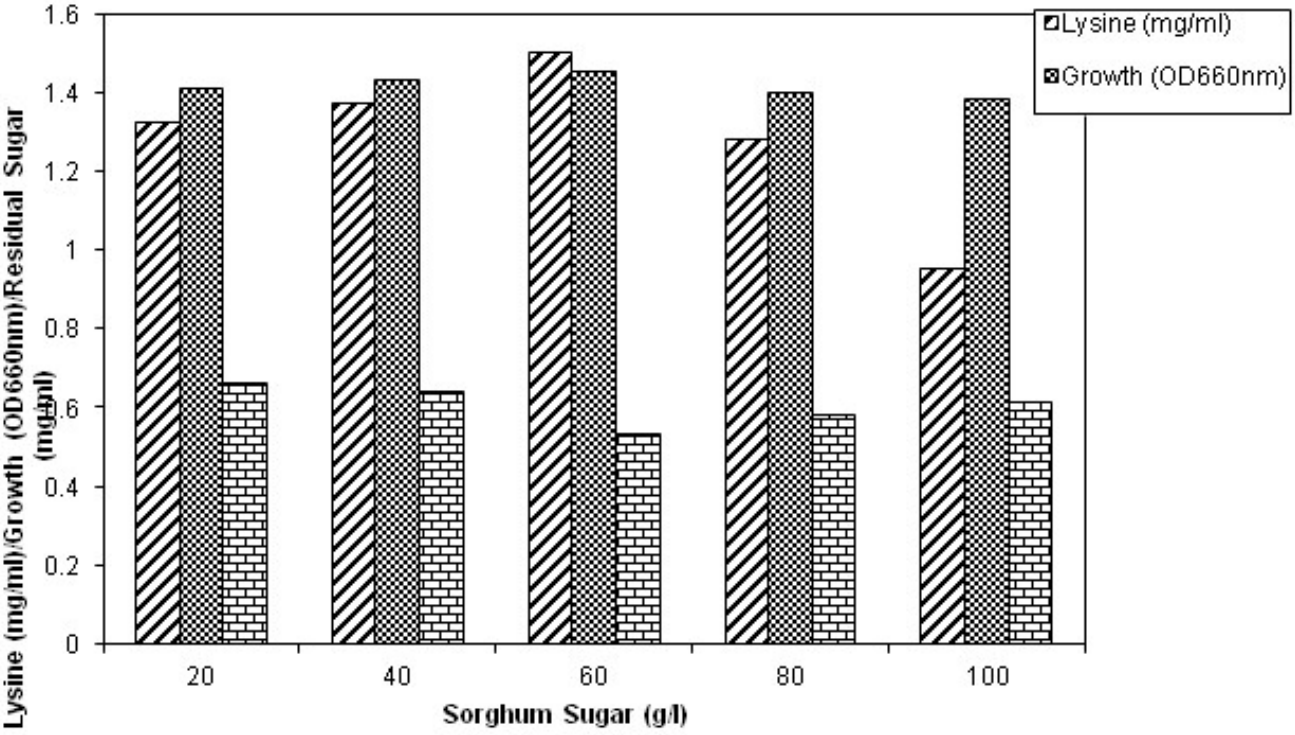

Figure 7: Effect of Varying Concentrations of Sorghum Sugar on Lysine Production by *Bacillus pumilus* SS16

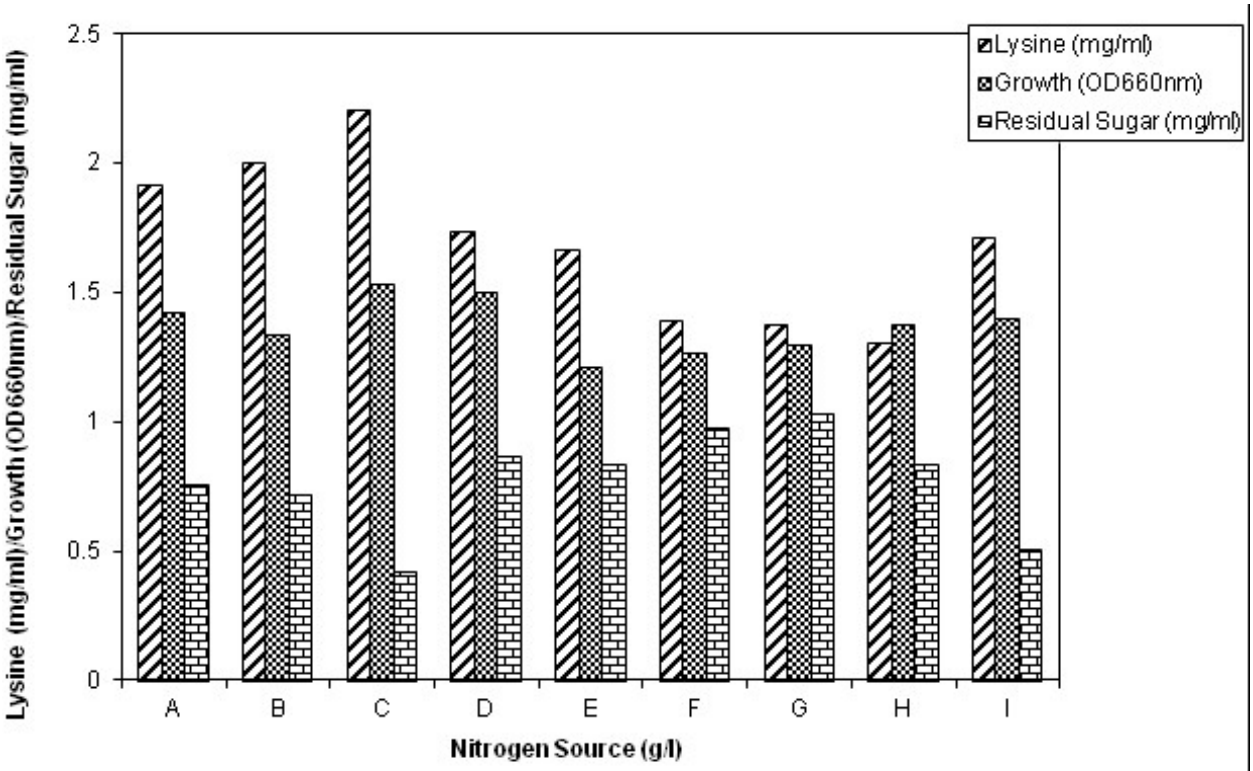

Figure 8: Effect of Nitrogen Sources on Lysine Production by *Bacillus subtilis* PR13: A, Peanut Meal; B, Bambara Nut; C, Soya Bean Meal; D, Cowpea Meal; E, Defatted Peanut Meal; F, Defatted Bambara Meal; G, Defatted Soya Bean Meal; H, Defatted Cowpea me

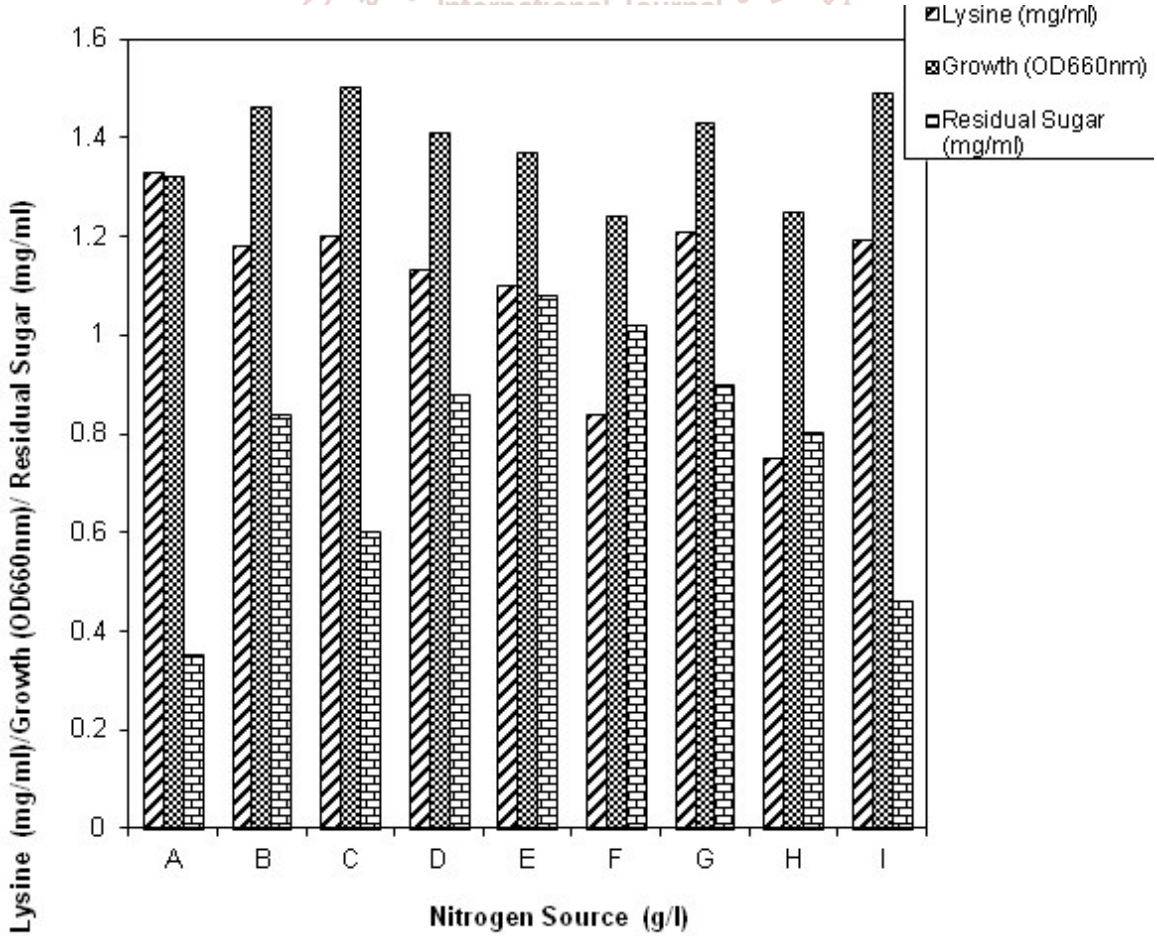

Figure 9: Effect of Nitrogen Sources on Lysine Production by *Bacillus subtilis* PR9: A, Peanut Meal; B, Bambara Nut; C, Soya Bean Meal; D, Cowpea Meal; E, Defatted Peanut Meal; F, Defatted Bambara Meal; G, Defatted Soya Bean Meal; H, Defatted Cowpea me

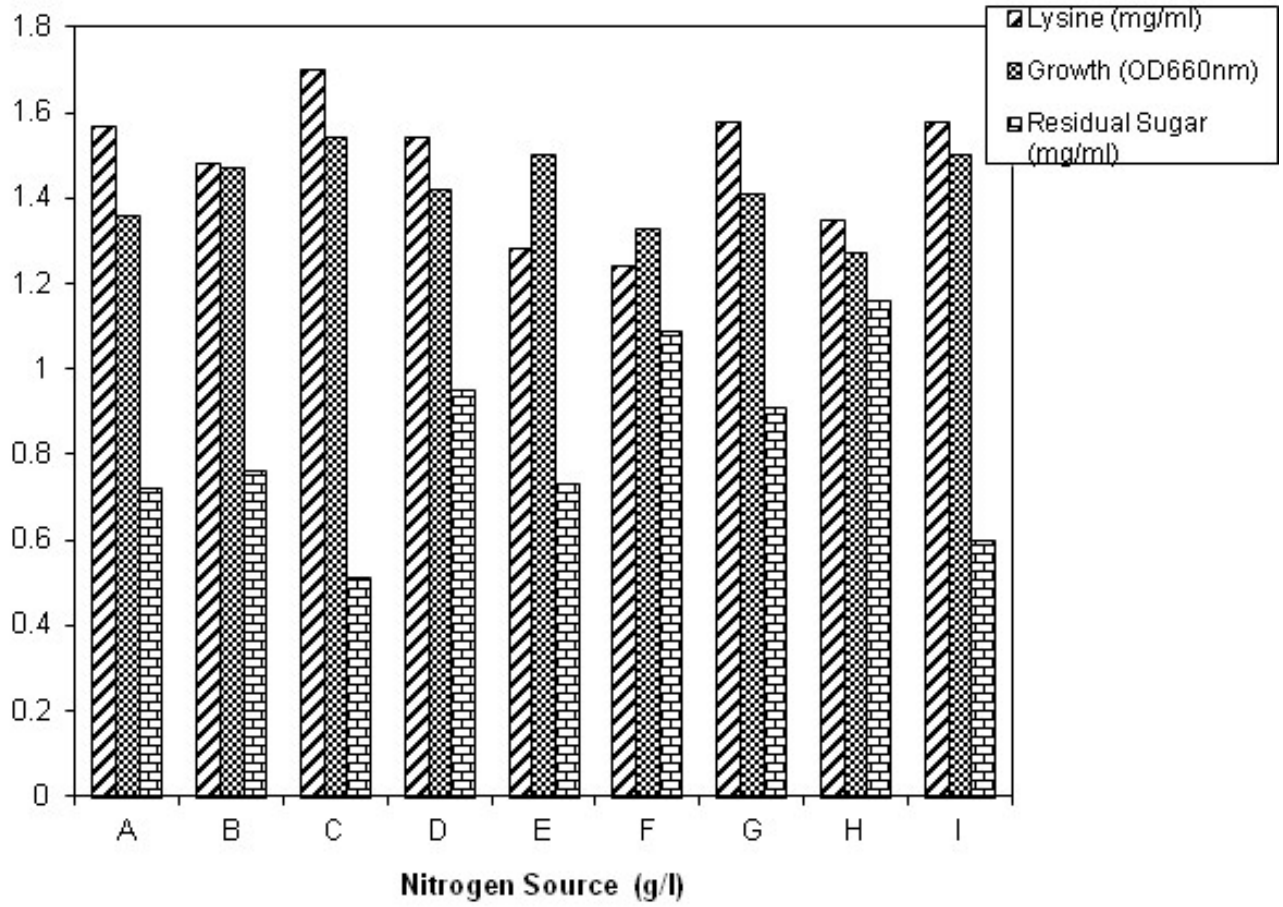

Figure 10: Effect of Nitrogen Sources on Lysine Production by *Bacillus pumilus* SS16: A, Peanut Meal; B, Bambara Nut; C, Soya Bean Meal; D, Cowpea Meal; E, Defatted Peanut Meal; F, Defatted Bambara Meal; G, Defatted Soya Bean Meal; H, Cowpea meal; I, A

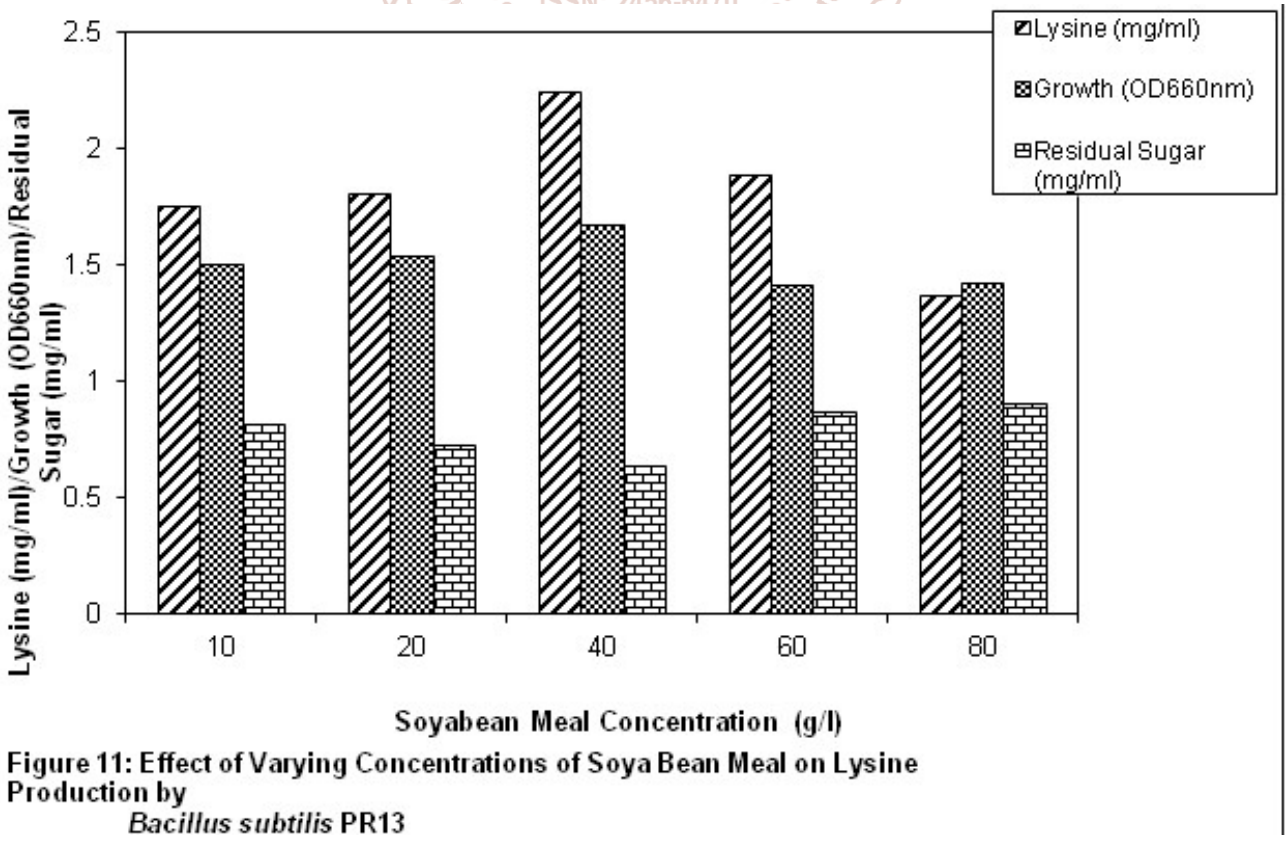

Figure 11: Effect of Varying Concentrations of Soya Bean Meal on Lysine Production by *Bacillus subtilis* PR13

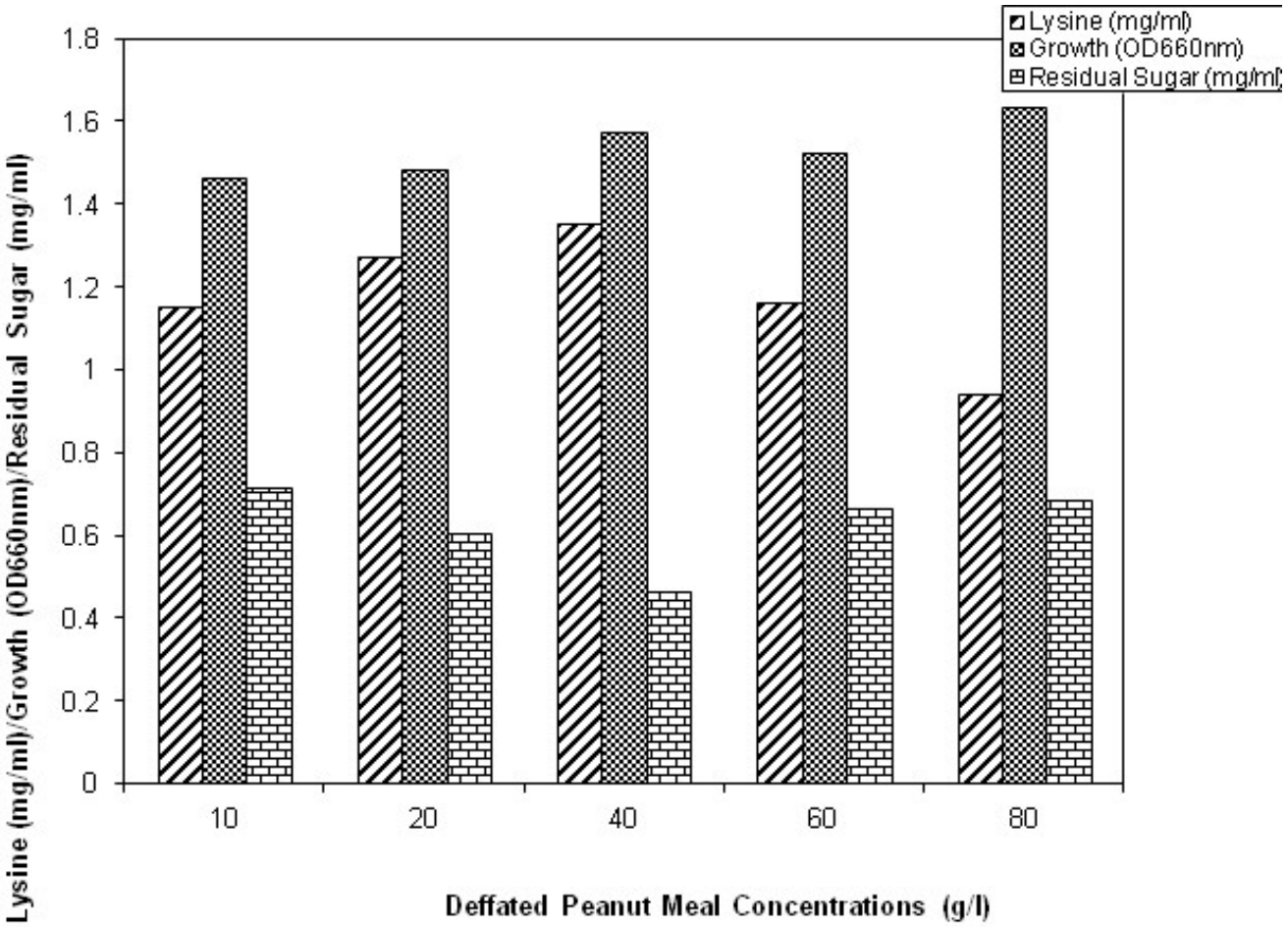

Figure 12: Effect of Varying Concentrations of Defatted Peanut Meal on Lysine Production by *Bacillus subtilis* PR9

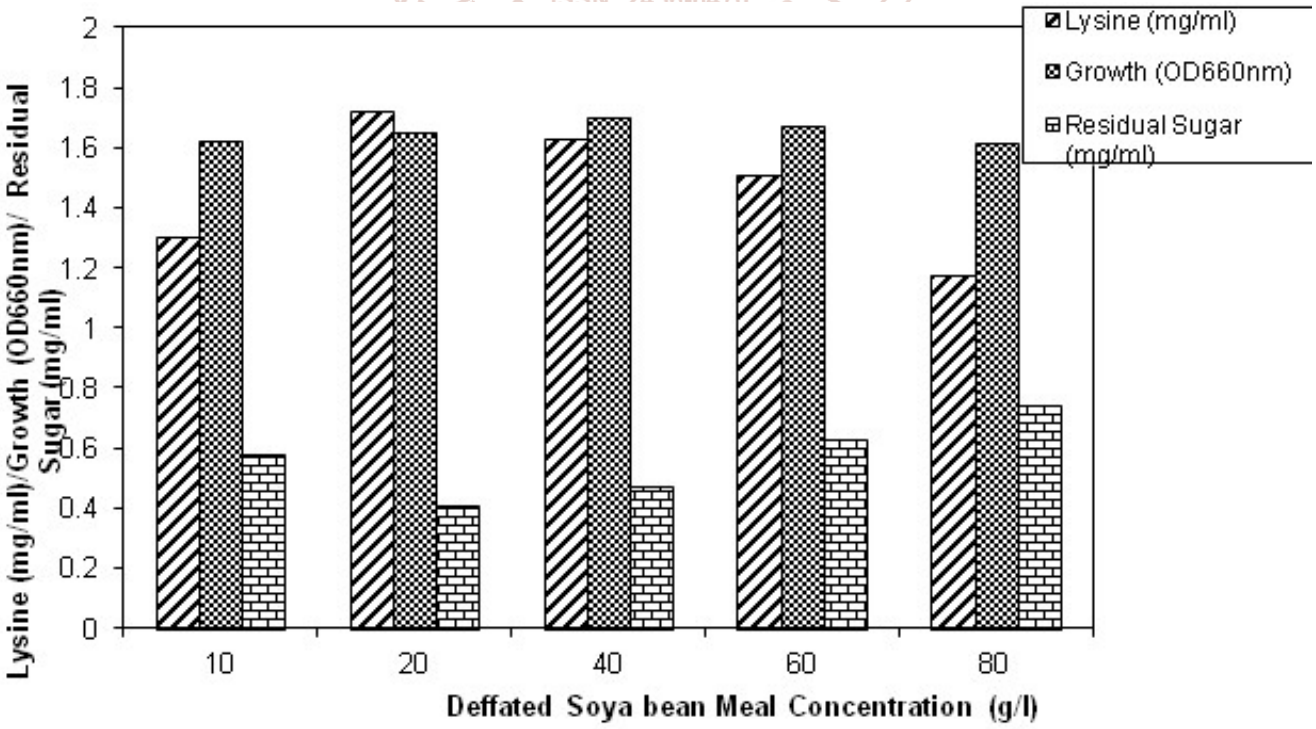

Figure 13: Effect of Varying Concentrations of Deffated Soya bean Meal on Lysine Production by *Bacillus pumilus* SS16

DISCUSSION

The study showed the susceptibility of the different starch sources to enzyme hydrolysis with varying reducing sugar production. Maize starch recorded the highest reducing sugar yield as compared with other starches. This is consistent with the report of Omemu *et al.* (2005) that maize starch had the highest reducing sugar yield as compared with other starches. The susceptibility of starch granules to digestion by amylase is dependent on starch source and length of amylase treatment (Okolo *et al*., 1995; Achi and Njoku-Obi 1992; Omemu *et al*., 2005). Sweet potato was hydrolysed in the study producing 2.1mg/ml of reducing sugar and this was contrary to the reports of Taniguich *et al*. (1982) and Okolo *et al*. (1995), that sweet potato starch was not easily hydrolyzed. Omemu *et al.* (2005) suggested that the ability of the crude amylase of *A. niger* AMO7 to hydrolyze the root starches especially cassava starch presented a remarkable property since these roots starches are abundantly available in the tropics. According to Anthony *et al*. (1996) and Oluwole *et al*. (1999) over 30 million tones of cassava are lost yearly since it is perishable after harvesting. Conversion of raw cassava by this enzyme means that some of the cassava could be used as raw materials by the starch industry for value added products. This will reduce wastage and improve economic gain.

The hydrolysates of millet for *B.subtilis* PR13 and sorghum for *B.subtilis* PR9and *B.pumilus* SS16 stimulated the highest growth and lysine production. Similar finding has been reported by Umerie *et al.* (2000) who reported that millet produced the highest lysine yield by *B. laterosporus*. Other workers used different starch hydrolysate for amino acid production. Nampoothiri and Pandey, (1999) reported that strain of *Brevibacterium* sp DSM utilized cassava starch hydrolysate and accumulated 21g/l of L-glutamic acid. Kubota *et al.* (1970), used sweet potato starch hydrolysate and obtained 5.20g/l of L-lysine HCl by threonine-valine auxotroph of *Brevibacterium lactofermentum*. Production strains of *C. glutamicum* and *Brevibacterium* species are able to grow and synthesize L-lysine in the fermentation medium with the paper hydrolyzate as the source of monosaccharides. The production of 20–24g of lysine per liter was achieved in media where hydrolysate was supplemented with saccharose that permited the sufficient growth with the simultaneous initiation of the production of L-lysine (Pelechova *et al*., 1983). Smekal *et al.,* (1983) reported lysine production in the range 36-44g/l when carbon sources which included hydrolysates of cereal starch were utilized by *Corynebacterium glutamicum*. Pharm *et al*.(1989), reported that lysine yield increased 1.5 fold to 16.9 g/l when sugar cane juice enriched with coconut water was used by a homoserine auxotroph, derived from *Corynebacterium glutamicum* ATTCC 13032.

Results from the study showed that sweet potato hydrolysate stimulated the lowest lysine production in *B.subtilis* PR13 and *B.pumilus* SS16 and this was similar to the findings of Shah and Hameed (2004).They recorded low lysine production (15 to 28g/l) in a medium containing starch hydrolysate using *Corynebacterium glutamicum*. They suggested that starch hydrolysates have relatively low content of monosaccharide, therefore the quantity of lysine that would be produced will be reduced compared to the use of glucose. Javed *et al.*(2011), observed that bacterial cells grown on 20g/l of corn steep liquor had higher quantities of lysine as compared to sucrose(3.57g/l) and acetate(14.82g/l) because corn steep liquor is a rich source of glucose and other growth enhancing products that can accelerate growth and product yield of microorganisms. Liu (1986) studied optimization conditions of different parameters for lysine production with cane molasses by *Brevibacterium* species and achieved high yield after optimization.The results of the study showed that 6% millet for *Bacillus subtilis* PR13 and 6% sorghum for *Bacillus subtilis* PR9 and *Bacillus pumilus* SS16 stimulated the highest amount of lysine. The finding is not in agreement with the report of other workers. Wang *et al.*(1991) reported that 9 and 16% initial sugar for molasses and sugar media respectively were optimum for lysine production. In a separate study conducted by Roy and Chatterjee (1989) they observed that 8% of glucose concentration produced the highest amount of glutamic acid respectively. Nampoothiri and Pandey (1995), reported that maximum yield of glutamic acid (6.86 mg/ml) was obtained when 2% glucose medium was fermented for 48h by a *Brevibacterium* species. Also, Ekwealor and Obeta (2005) in their study observed that *B. megaterium* SP14 recorded the highest amount of lysine when 8.0% (w/v) glucose was used as source of carbon. Hadj-Sassi *et al.*(1988), reported the influence of initial concentration of glucose from 80 to 233 g/l on the production of L-lysine by *Corynebacterium* species in batch and feed batch culture. The maximum conversion rate in L-lysine was obtained at 165g/l and the best specific production rate of L-lysine was observed at 65g/l of glucose. Pfefferle *et al.*(2003), reported that lysine was exclusively produced in a bioprocess employing *Corynebacterium glutamicum* which generates a large amount of L-lysine from cane sugar and corn starch. Decrease in lysine production at higher carbon source (millet and sorghum) concentrations might be due to feed back inhibition of higher levels of carbon source that reverses the glycolytic pathway and inhibits the utilization of glucose (Ikeda, 2003). Pham *et al*. (1992), used sugarcane juice, molasses, banana, cassava and coconut water as carbon sources for methionine production. Glucose is the most widely used carbon source (Kase and Nakayama, 1974; Chattopadhyay *et al*., 1995 a, b), but Banik and Majumdar (1974) reported maltose as the best carbon source for the production of methionine.

The stimulation of lysine production by soyabean meal in *B.subtilis*PR13 and *B.pumilus* SS16 is similar to the report of Adnan *et al.*(2011).They studied the selection of subtrates for L-lysine production by *Brevibacterium linens* DSN 20158 and observed maximum L-lysine production (2.213g/kg) with soyabean meal. There was no general defined medium for lysine production by different microbial strains(Pandey *et al.,*(2000).Every microorganism has its own characteristic physico-chemical and nutritional requirements for L-lysine production. In view of the economical use of the L-lysine, cheap medium formulation was kept in mind. Therefore cheap solid substrate i.e. Soybean meal was used for the maximum production of L-lysine as reported by Ekwealor and Orafu (2003), because it is a good source of proteinaceous nitrogen and other nutritious substances essential for bacterial growth. Umerie *et al.*(2000) studied the effects of various nitrogen sources and was able to observe that defatted soyabean meal stimulated the highest amount of lysine. Smekel *et al.*(1982), studied the production of L-lysine with the strain of *Brevibacterium flavum* and *Corynebacterium glutamicum*, using saccharose technology and non standard nitrogen sources such as hydrolysates of extracted rap, flax and cotton plant crush and hydrolysates

of fodder yeast. Using these nitrogen sources the production in a range from 36 to 45g L-lysine per liter was achieved. Again Smekel *et al.* (1984), studied the biosynthesis of L-lysine in *Corynebacterium glutamicum* and *Brevibacterium flavum* using media with a hydrolysate of phosphorcarpus flour, they observed a lysine yield of 44 and 30 g/l respectively.

It is concluded from the present studies that the production of L-lysine from *Bacillus* species can be substantially enhanced by optimizing the culture medium. Carbon and nitrogen sources have also been found to have influential role in the amino acid production.

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