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Optimization of Cellulase and Amylase Production by *Bacillus* Strain Isolated from Cultured Fish Intestine

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ABSTRACT
The optimization of some culture conditions for cellulase and amylase production by Bacillus sp. previously isolated from the gut of a farmed freshwater fish (Clarias gariepinus) was carried out. The varied culture parameters assessed were incubation temperature (28, 30, 37, 40 and 45^{o}C), medium pH (4, 5, 6, 7, 8, 9 and 10), additional carbon sources (fructose, sucrose, lactose, glucose, cellulose and starch) and nitrogen sources (yeast extract, urea, ammonium sulphate and peptone). The cellulase and amylase activities of each culture were measured by determining the amount of reducing sugars liberated by using a Dinitrosalicyclic acid (DNS) method and carboxymethyl cellulose and starch as substrate respectively. The optimum conditions observed for cellulase production were incubation temperature 40^{o}C, medium pH 7.0, 1.0% glucose as additional carbon source and yeast extract as nitrogen source. The maximum incubation temperature, medium pH, additional carbon source and nitrogen source observed for amylase production were 37^{o}C, 7.0, glucose and urea respectively. This bacterial isolate has potential that could be commercially exploited to assist in cellulose and starch degradation in various biotechnological applications.
Keywords: *Fish intestine, bacteria, enzyme production, optimization*

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INTRODUCTION

Cellulose is a polymer of glucose residues connected by β 1, 4 linkages. It is the primary structural material of plant cell wall and the most abundant carbohydrate in nature (Saha *et al*., 2006). The enzyme that hydrolyses the beta 1, 4 glycosidic bonds in the polymer to release glucose units is called cellulase (Nishida *et al*., 2007). Cellulolytic enzymes play an important role in natural biodegradation processes in which cellulolytic bacteria, fungi, actinomycetes and protozoa efficiently degrade materials. Cellulases have a wide range of enormous potential applications in biotechnology. They

are used in the production of fermentable sugars and ethanol (Levy *et al*., 2002; Vanlwyk and Mohulatsi, 2003), organic acids (Luo *et al*., 1997), detergents and other chemicals (Oksanen *et al*., 2000).

They are also used in pulp and paper industry (Oksanen *et al*., 2000), in texile industry (Cavaco-Paulo and Gubitz, 2003; Miettinen- Oinonen *et al*., 2004; Nierstrasz and Warmoeskerken, 2003), in improving digestibility of animal feeds (Ishikuro, 1993) and in food industry (Penttila *et al*., 2004; Urlaub, 2002). Bacteria present an attractive potential for the exploitation of cellulases due to their rapid growth rate, enzyme complexity and extreme habitat variability (Maki *et al*., 2009). Bacteria inhibit a wide variety of environmental niches which produce cellulolytic strains that are extremely resistant to environmental stresses (Maki *et al*., 2009).

Starch is one of the most widely available plant polysaccharides and a major ingredient of the feed for fishes. It is hydrolyzed to its constituent sugar and oligosaccharides in the digestive tract of the fish (Sugita *et al*., 1997). The enzyme amylase is widely distributed in the intestinal tract of freshwater fish and plays an important role in the digestion of starch (Sugita *et al*., 1997; Ariole *et al*., 2014). Alpha-amylase (EC3.2.1.1) cleaves internal α-1, 4-glycosidic linkages in starch to produce glucose, maltose or dextrins and glucoamylase (EC3. 2.1.3) and α-1, 6-glycosidic linkages to release glucose from the non-reducing ends of starch (Xiao *et al*., 2006). Amylases can be obtained from several sources such as plant, animal and microorganisms (Kathiresan and Manivannan, 2006). Microbial production of amylase is more efficient than that of other sources as the technique is easy, fast and cost effective. It can be modified to obtain enzymes of desired characteristics (Ashwini *et al*., 2011).

The potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Akpan *et al*., 1999; Pandey *et al*., 2000; Abu *et al*., 2005). With the advent of new frontiers in biotechnology, amylases have wide potential application in a number of industrial processes such as in the food, paper, textiles, detergent, baking, brewing, pharmaceuticals, fruit juices, sweeteners and spot remover in dry cleaning (Qader *et al*., 2006; Alva *et al*., 2007; Kammoun *et al*., 2008; Bairagi *et al*., 2002; Arikan, 2008; Al Tameemi, 2010; Akpa *et al*., 2004). These uses have placed greater stress on increasing indigenous amylase production and search for more efficient processes (Aiyer, 2005).

The bacterial flora within the GI tract of fish shows very broad and variable

enzymatic potential and these enzymatic masses may interfere positively in the digestive process of fish. Fish gut bacterial isolates have been demonstrated to break down cellulose (Saha and Ray, 1998; Bairagi *et al*., 2002; Ghosh *et al*., 2002a; 2010; Saha *et al*., 2006; Mondal *et al*, 2008;) and starch (Banerjee and Ray, 2013; Ariole *et al*., 2014). These intestinal bacterial isolates have potentials that could be exploited to assist in cellulose and starch degradation in various industrial processes. The search for extracellular enzyme-producing beneficial gut bacteria to be used as probiotics for the culturable freshwater fish species and for other industrial purposes will continue to be of interest.

Although a large number of microorganisms can degrade cellulose, only a few of them produce significant quantities of free enzymes capable of completely hydrolyzing crystalline cellulose (Koomnok, 2005). Cellulase yields appear to depend upon a complex relationship involving a variety of factors such as temperature, carbon sources, medium additives, aeration, presence of inducers, incubation period, cellulose quality and pH of the medium (Immanuel *et al*., 2006). The demands for more stable, highly active and specific enzymes will continue to grow (Sethi *et al*., 2013) and hence, isolation and characterization of cellulase producing bacteria will continue to be an important aspect of biofuel research (Hirasawa *et al*., 2006).

Several physical and chemical parameters such as pH, temperature, incubation time, carbon source and nitrogen source affect the enzyme production and the enzyme activity (Sudharhsan *et al*., 2007). Optimization of growth condition is a prime step in using microorganisms in fermentation technology (Kathiresan and Manivannan, 2006). The high cost of enzyme production (due to use of pure chemical in production) coupled with low enzyme activities, limits its industrial use. Therefore, efforts are needed to economize cellulase and amylase production by media optimization (Kalra *et al*., 2008). Furthermore, enzyme production is closely controlled in microorganisms and to improve its productivity, these controls can be ameliorated. Therefore, the aim of the present work was to optimize the culture conditions for cellulase and amylase production from *Bacillus* sp. previously isolated from cultured *Clarias gariepinus* intestine (Ariole *et al*., 2014).

MATERIALS AND METHODS

Source of Cellulolytic and Amylolytic Bacterium (*Bacillus* sp.):

Bacillus sp. was previously isolated from fish, *Clarias gariepinus*, intestine (Ariole *et al*., 2014) and maintained as part of culture collection in Microbiology

Laboratory, University of Port Harcourt, Nigeria.

Cellulase and Amylase Production:

The production medium of Sethi *et al*. (2013) containing (g/L): cellulose 10.0, peptone 0.75, $FeSO_4$ 0.1, KH_2PO_4 0.5, $MgSO_4$ 0.5 and pH 7.0 was used for cellulase production while the production medium of Ashwini *et al*. (2011) containing (g/L): starch 10, peptone 10, yeast extract 20, KH_2PO_4 0.05, $MnCl_2.4H_2O$ 0.015, $MgSO4.7H_2O$ 0.25, $CaCl_2.2H_2O$ 0.05, $FeSO_4$. $7H_2O$ 0.01 and pH 7.0 was used for amylase production. One millilitre of a 24 h nutrient broth culture of the *Bacillus* sp. was inoculated into 100ml of production medium in 500 ml Erlenmeyer flask. The inoculated flasks were then incubated for a period of 48 h at 37^{o}C. The culture medium was centrifuged at 5000 rpm for 15 min to obtain the crude extract which was used as enzyme source.

Cellulase Assay:

The amount of reducing sugar released by the cellulolytic bacterium was measured by Dinitrosalicyclic acid (DNS) method of Miller (1972) described by Otajevwo and Aluyi, (2011). A reaction mixture which consisted of 0.2 ml of crude enzyme solution and 1.8 ml of 0.5 % carboxymethyl cellulose (CMC) in 50 mM sodium phosphate buffer (pH 7) was incubated at 37^{o}C in a shaking water bath for 30 min. The reaction was terminated by adding 3 ml of DNS reagent. The colour was then development by boiling the mixture for 5 min. The optical density (OD) was measured at 540 nm against a blank containing all the reagents except the crude enzyme using spectrophotometer. A unit (U) of cellulase activity was defined as the amount of cellulase required to catalyse the liberation of reducing sugar equivalent to 1µmol of D-glucose per minute under the assay conditions.

Amylase Assay:

The amount of reducing sugar released by the amylolytic bacterium was measured by Dinitrosalicyclic acid (DNS) method of Miller (1972) described by Otajevwo and Aluyi (2011). Amylase assay was done by using mixture consisting of one millilitre substrate solution (1% soluble starch in 50 mM phosphate buffer, pH 7) and 0.1 ml enzyme solution. The mixture was then incubated for 10 min at 37^{o}C. The reaction in the mixture was stopped by adding 2.0 ml of DNS reagent and then kept in boiling water bath for 5 min. After cooling at room temperature, the optical density (OD) was measured at 540 nm against a blank containing all the reagents minus the crude enzyme using spectrophotometer (Spectronic N21). One unit (U) of amylase activity was defined as the amount of amylase required to catalyse the liberation of reducing sugar equivalent to 1µmol of D-

glucose per minute under the assay conditions.

Optimization of Culture Conditions for Catalase and Amylase Production

Effect of Temperature

Bacillus sp. was cultured at temperatures ranging from 25 to 45^{o}C to select the optimum temperature for maximum cellulase and amylase production using cellulase production medium and amylase production medium respectively while the other parameters were unaltered. Incubation was for 48 h. Each culture broth was harvested by centrifugation at 5000 rpm for 15 min. The supernatant collected from each culture was used as crude enzyme and was assayed for the respective enzyme activity.

Effect of Medium pH

The effect of pH of the cellulase production medium and amylase production medium on cellulase and amylase production respectively was performed by varying pH of each medium from 5 to 10 while keeping the remaining parameters constant. Incubation was for 48 h at 37^{o}C and each crude enzyme obtained after harvesting each culture broth was assayed for the respective enzyme activity.

Effect of Carbon Sources

The effect of additional carbon sources on enzyme production by *Bacillus* sp. was analysed by supplementing the cellulase production medium with different carbon sources (fructose, starch, sucrose, lactose and glucose) at a concentration of 1% (w/v). Fructose, cellulose, glucose, lactose and sucrose at a concentration of 1% (w/v) were added into amylase production medium in 500 ml Erlenmeyer flasks containing 100 ml of liquid medium. Incubation was for 48 h at 37^{o}C and each crude enzyme obtained after harvesting each culture broth was assayed for the respective enzyme activity.

Effect of Nitrogen Sources

The effect of nitrogen sources on cellulase and amylase production was studied by incorporating different nitrogen sources (urea, ammonium sulphate, peptone and yeast extract) in each production medium at a concentration of 1% (w/v). Incubation was for 48 h at 37^{o}C and each crude enzyme obtained after harvesting each culture broth was assayed for the respective enzyme activity.

Statistical Analysis: Standard deviations for each of the experimental results were calculated using Excel Spreadsheets, with Microsoft excel software. Differences between treatments were examined for significance by one-way ANOVA and P = 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

In this study, we assessed the effects of culture conditions on cellulase and amylase producing ability of *Bacillus* sp.

previously isolated from the intestine of a freshwater fish (*Clarias gariepinus*) (Ariole *et al*., 2014). Many microorganisms such as bacteria and fungi are known as potential producers of extracellular cellulase and amylase (Irfan *et al*., 2012; Sethi *et al*., 2013; Otajevwo and Aluyi, 2011; Shahriarinour *et al*., 2011; Fossi *et al*., 2009; Ibatsam *et al*., 2011; Ashwini *et al*., 2011; Sidkey *et al*., 2010). Furthermore, bacteria in the digestive tract of aquatic animals participate with their enzymes in the process of degradation of nutrients (Banerjee and Ray, 2013); Ariole and Kalu, 2014; Sugita *et al*., 1997).

The effect of incubation temperature on cellulase production is shown in Fig. 1. The temperature of 40^{o}C was found to be optimum with maximum of 0.53 U/ml cellulase activity.

The enzyme production decreased beyond 40^{o}C. Similarly, the maximum cellulase production by *Serratia marscens*, *E. coli*, *Bacillus subtilis* and *Pseudomonas fluorescens* (Sethi *et al*., 2013) and *Bacillus subtilis* and *Bacillus circulans* (Ray *et al*., 2007), was at 40^{o}C. These results are different from the finding of Otajevwo and Aluyi (2011) who recorded 35^{o}C as optimal for cellulolytic activities of *Serratia* spp., *Bacillus subtilis* and *Bacillus circulans*. Also different are the reports of Li *et al*. (2008) and Hirasawa *et al*. (2006) who recorded 50^{o}C as optimal for cellulase production by *Bacillus subtilis* and *Bacillus agaradherens* JAM- Ku -23. These observed differences in optimal temperature for cellulase production may be as a result of differences in source, type and species of the cellulolytic organisms.

The effect of incubation temperature on amylase production is shown in Fig. 2. The temperature of 37^{o}C was found to be optimum with maximum of 0.52 U/ml amylase activity. The enzyme production decreased beyond 37^{o}C. This finding is contrary to that of Ashwini *et al*. (2011) who reported that amylase production by *Bacillus* sp. *marini* was optimum at 40^{o}C. The decrease in enzyme production beyond certain temperature plays a major role in enzyme production. It has been reported that temperature influences secretion of extracellular enzymes by changing the physical properties of the cell membrane (Sahu and Martin, 2011). The effects of pH on cellulase and amylase production are presented in Figures 3 and 4 respectively. Maximum cellulase and amylase activities of 0.79 U/ml and 0.42 U/ml respectively, were observed at pH 7.0. Cellulase and amylase activities declined beyond pH 7.0. Similarly, maximum cellulase activity was observed at pH 7.0 for *Serratia marscens*, *E. coli*, *Bacillus subtilis* and *Pseudomonas fluorescens* (Sethi *et al*., 2013) and *Bacillus* sp. *marini* (Ashwini *et al*., 2011). These results imply that initial pH

of culture medium affects the rate of enzyme production.

The effects of additional carbon sources on cellulase and amylase production are shown in Figures 5 and 6 respectively. The use of fructose, starch, sucrose, lactose or glucose as additional carbon source positively affected cellulase production. The maximum cellulase activity (0.92 U/ml) was observed when glucose was used as additional carbon source. Fructose and glucose also influenced amylase production. The maximum amylase activity (0.71 U/ml) occurred when glucose was used as additional carbon source. However, cellulase, lactose and sucrose caused a reduction in amylase production in comparison with the control (Fig. 6). This reduction in enzyme production in the presence of sugars as carbon sources could be due to catabolite repression by readily available carbon sources in the medium (Kiran *et al*., 2008).

The effects of nitrogen sources on cellulase and amylase production are shown in Figures 7 and 8 respectively. Among the various nitrogen sources tested, yeast extract was found to be the best nitrogen source for cellulase production while urea was the best for amylase production. However, the maximum cellulase and amylase activities were observed when ammonium sulphate was employed as nitrogen source for cellulase production (Sethi *et al*., 2013) and yeast extract for amylase production (Ashwini *et al*., 2011). Production of extracellular enzyme has been reported to be sensitive to repression by different carbohydrate and nitrogen sources (Sethi *et al*., 2013). The results obtained in this study suggest that cellulase and amylase production by *Bacillus* sp. is strongly influenced by medium components as well as cultural parameters.

CONCLUSION

This study reports the optimization of some culture conditions for production of cellulase and amylase by *Bacillus* sp. previously isolated from a freshwater fish (*Clarias gariepinus*) intestine. Cellulase production was found to be optimal in the presence of glucose as additional carbon source and yeast extract as nitrogen source. The optimal temperature and pH for cellulase production were 40^{o}C and 7.0 respectively. The optimum conditions observed for amylase production were temperature 37^{o}C, pH 7.0, glucose as additional carbon source and urea as nitrogen source. This indigenous bacterial isolate has potential that could be commercially exploited to assist in cellulose and starch degradation in various industrial processes.

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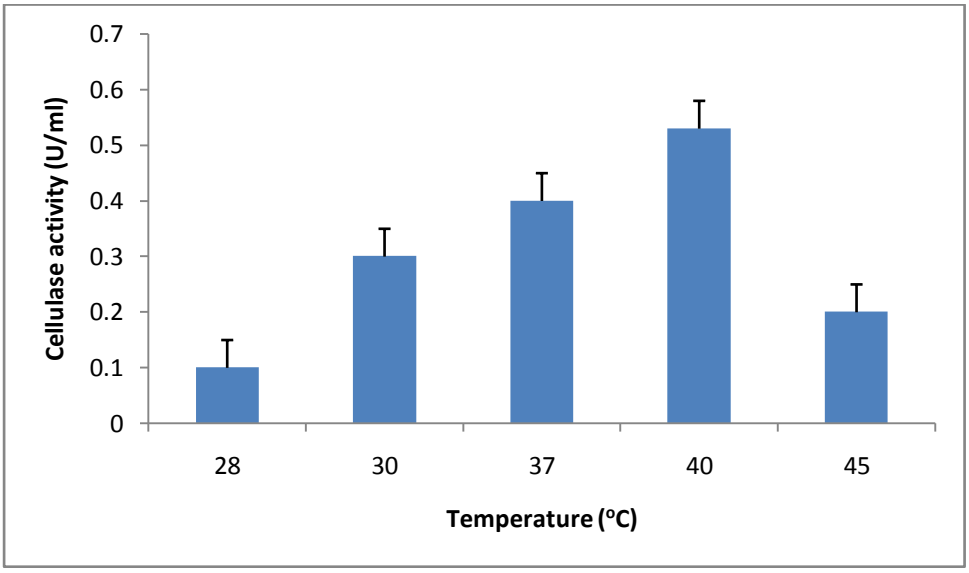

Figure 1: Effect of incubation temperature on cellulase production by *Bacillus* sp.

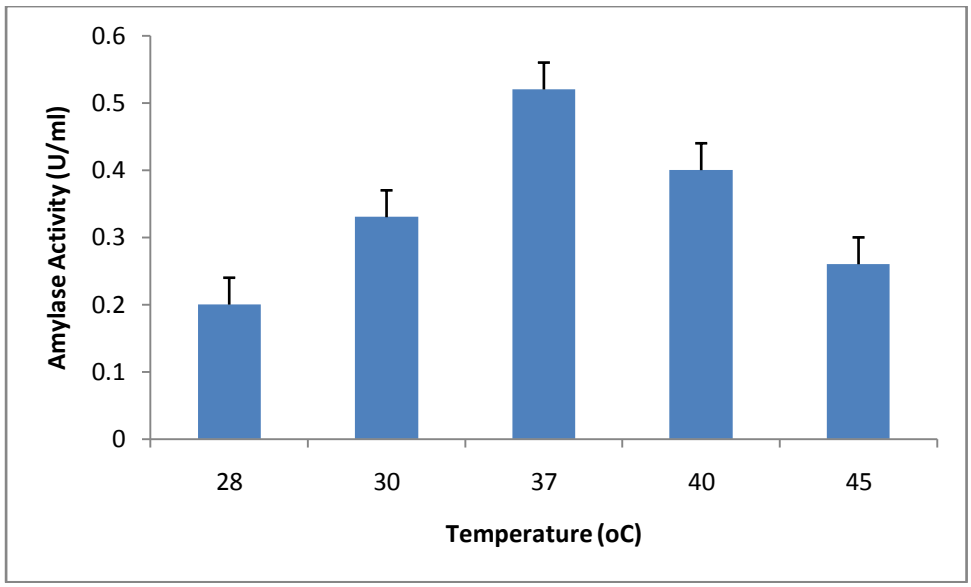

Figure 2: Effect of incubation temperature on amylase production by *Bacillus* sp.

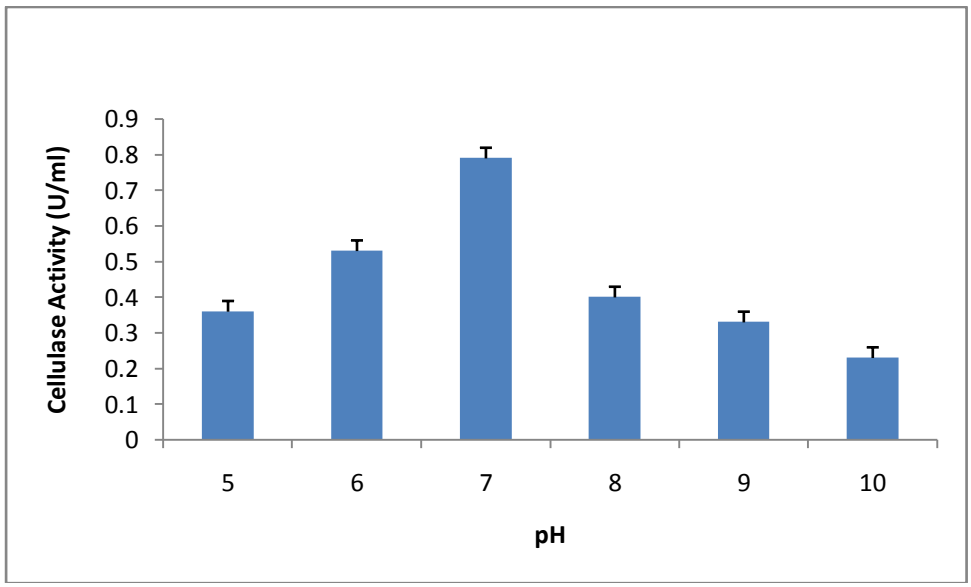

Figure 3: Effect of medium pH on cellulase production by *Bacillus* sp.

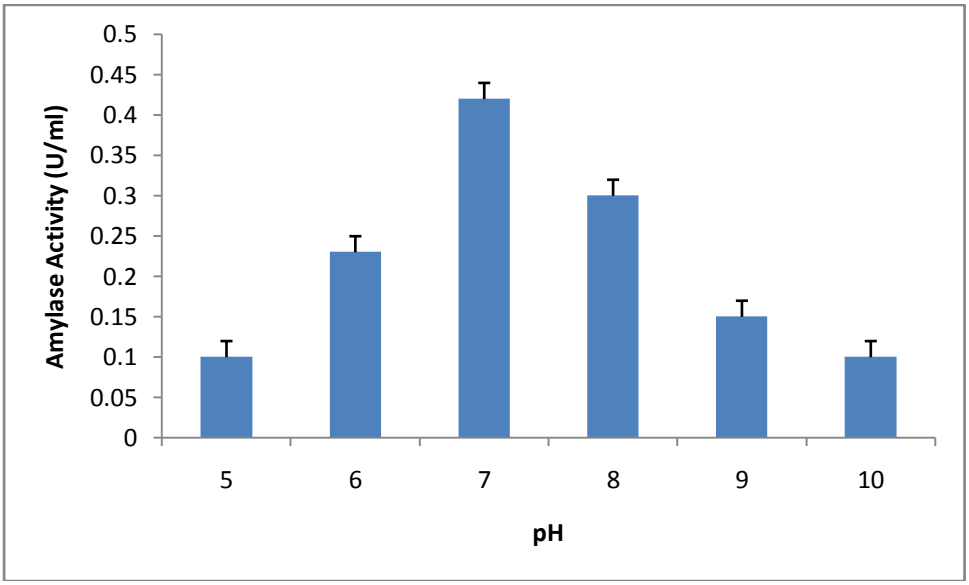

Figure 4: Effect of medium pH on amylase production by *Bacillus* sp.

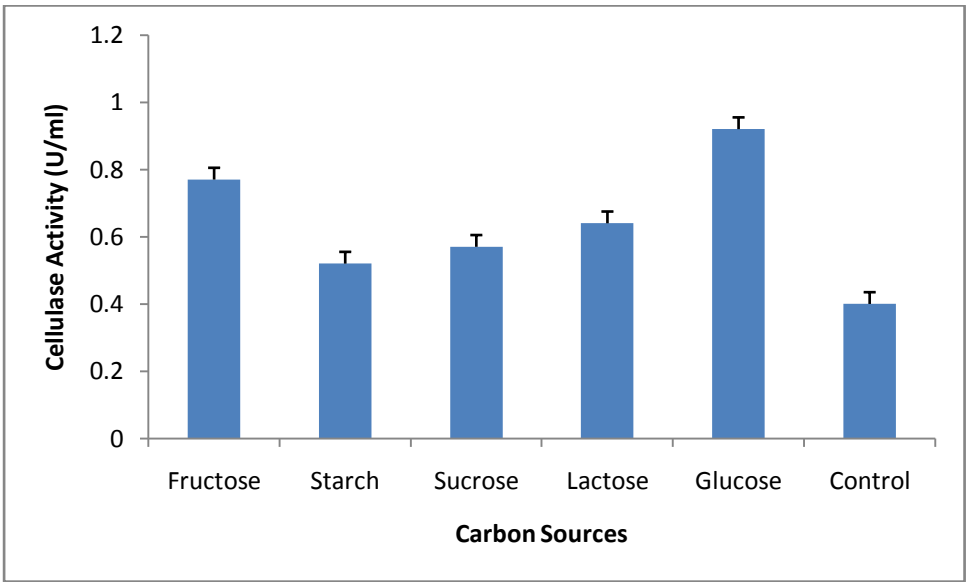

Figure 5: Effect of additional carbon sources on cellulase production by *Bacillus* sp.

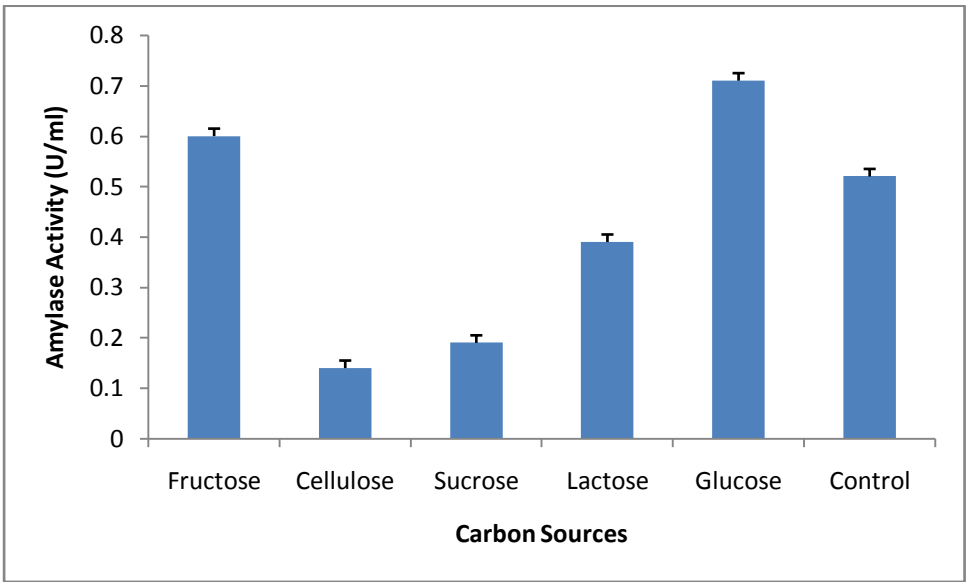

Figure 6: Effect of additional carbon sources on amylase production by *Bacillus* sp.

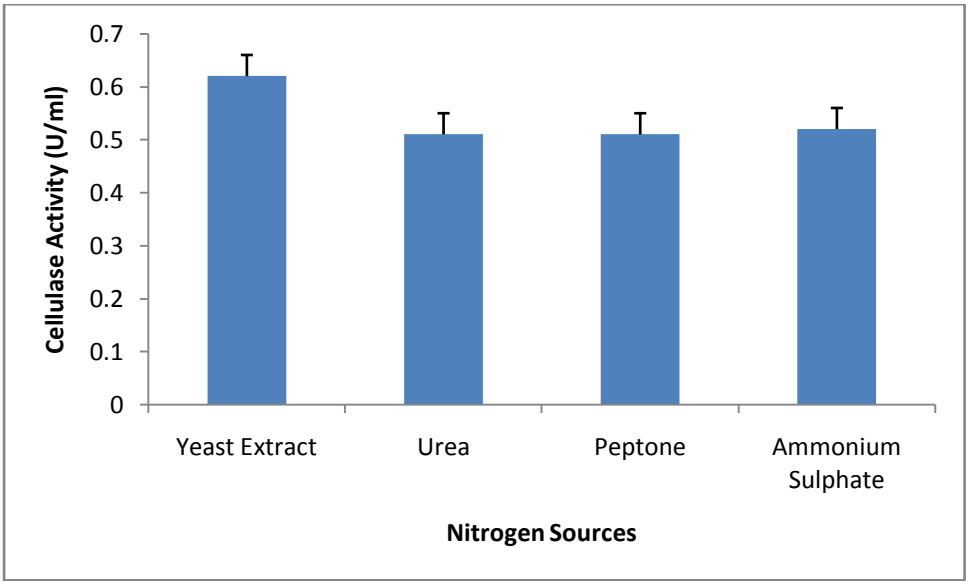

Figure 7: Effect of nitrogen sources on cellulase production by *Bacillus* sp.

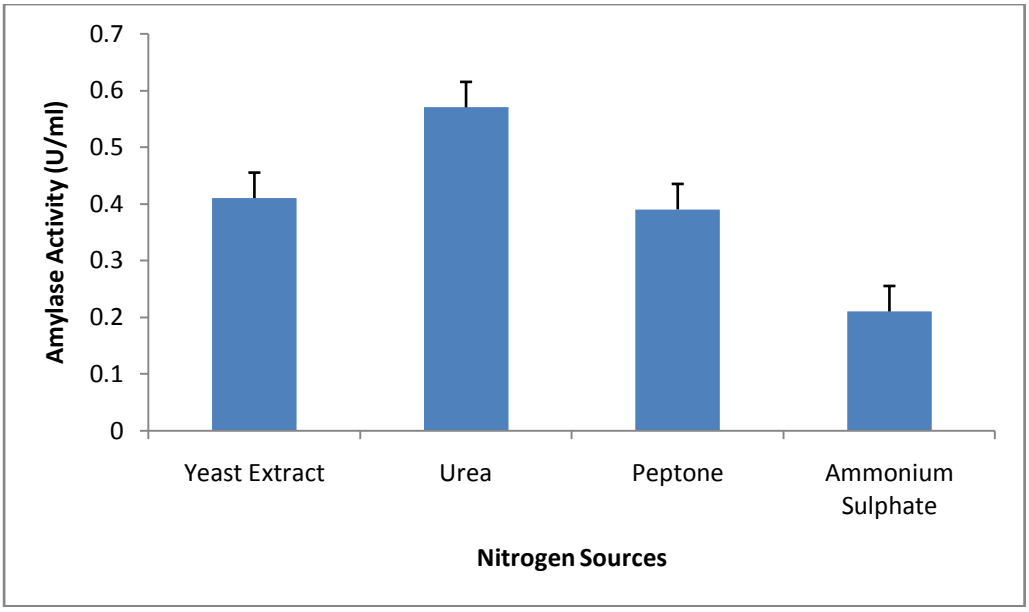

Figure 8: Effect of nitrogen sources on amylase production by *Bacillus* sp.