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PRODUCTION AND OPTIMIZATION OF A-AMYLASE FROM *ASPERGILLUS NIGER* USING POTATO PEEL AS SUBSTRATE

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ABSTRACT

The present study is concerned with the production and characterization of α-amylase by *Aspergillus niger* in solid-state fermentation using food waste as substrate. Various cultural conditions such as incubation period, incubation temperature, pH of the medium, moisture level and inoculum size were optimized for maximum α-amylase yield. The maximum activity of enzyme (1262.27 ± 2.11 U/g) was recorded after 72 h of incubation at 30°C temperature, pH 5 with 5% moisture level and inoculum size. Among different nitrogen and carbon sources evaluated, peptone (1.5%), NH_4NO_3 (0.75%) and soluble starch (1.25%) gave maximum α-amylase production under optimized conditions. Under all the optimized culture conditions, the maximum enzyme production was 1298.12 ± 2.14 U/g.

Key words: amylase, *A. niger*, potato peel, solid state fermentation

INTRODUCTION

Alpha amylase (endo-1,4-α-D-glucan glucanohydrolyase E.C. 3.2.1.1) is an extracellular enzyme that randomly cleaves 1,4-α-D-glucosidic bonds between adjacent glucose molecules in the linear amylose chain and eventually produces a range of products such as glucose and maltose units (Ramachandran *et al*., 2004; Sivaramakrishnan *et al*., 2007; Erdal and Taskin, 2010). Alpha amylases are produced by plants, animals and microorganisms, however, for industrial production, microbial (i.e. fungal and bacterial) sources are used, due to some benefits such as low cost, reliability, less time and space needed for enzyme production (Burhan *et al*., 2003). Alpha amylases are extensively used in textile, confectionary, baking, brewing, sugar, alcohol, paper coating, starch, pharmaceutical, syrup industries, digestive aid, detergents and for sewage treatment (Sivaramakrishnan *et al*., 2006; Dhanya *et al*., 2009; Lokeswari, 2010).

Filamentous fungi are being widely used due to their ability to produce large number of industrial enzymes. *Aspergillus niger* is a key group of microorganisms, which dominates in solid-state fermentation and is widely grown for production of amylases due to their properties such as ability to spread over and to penetrate inside the solid-substrate and good tolerance to low water availability (Manpreet *et al*., 2005; Abdullah *et al.,* 2011).

Traditionally, α-amylase was produced by submerged fermentation (SmF). In recent years, however, solid state fermentation has been utilized more and more for the production of α-amylase (Xu *et al*., 2008) due to advantages, such as simple technique, superior and high volumetric productivity, low capital investment, low catabolite repression, marginal end-product inhibition, low energy requirement, simple fermentation equipment requirement, less water output, better product recovery (Pandey *et al*., 2000; Gangadharan *et al*., 2006).

Several investigators have described the utilization of cheap and easily available food and agricultural wastes such as potato peel, wheat straw, wheat bran, coffee waste, banana waste and sugarcane bagasse as substrate in SSF for the production of α-amylase (Xu *et al*., 2008; Murthy *et al*., 2009). A variety of products such as, mashed potatoes, chips and fries etc are prepared from potatoes after peeling in processed food industries (Shukla and Kar, 2006; Schieber *et al*., 2009). These potato peels create severe disposal and pollution problems because these were considered as waste, discarded and permitted to rot. Potato peels also contain sufficient amount of nutrients like carbohydrates and

protein, to support the growth of microorganism. Therefore, these peels should be used as a substrate in SSF for α-amylase production (Shukla and Kar, 2006; Ajao *et al*., 2009). The aim of this study was the optimization of various process parameters for α-amylase production by *Aspergillus niger* through solid state fermentation using food waste as substrate.

MATERIALS AND METHODS

Chemical analysis of substrate: Potato peel used as substrate was obtained from Lays, Pepsi-cola International (Pvt) Ltd, Lahore, Pakistan. The moisture, ash, nitrogen and fat contents of potato peel were determined following the standard methods described in AOAC, (2005).

Microorganism and culture maintenance: A fungal strain of *Aspergillus niger* SM 24 was obtained from the microbiology laboratory of Food and Biotechnology Research Center (FB RC), PCSIR Laboratories Complex, Lahore. The culture was maintained on Potato-Dextrose-Agar (PDA) slants. The slants were grown at 30°C for 5 days and stored at 4°C.

Inoculum preparation: Ten ml of sterilized distilled water was added to a sporulated 5 days old PDA slant culture. An inoculum needle was used to dislodge the spore clusters under sterilized conditions and then it was shaken thoroughly to prepare homogenized spore suspension.

Solid-state fermentation: Twenty grams raw potato peel amended with 2 ml of mineral salt solution containing (g/l) KH_2PO_4 10, $MgSO_4$ 2.0, NaCl 2.0 and $MnSO_4$ 0.5 was taken in 250 ml cotton plugged Erlenmeyer flask, mixed homogenously and sterilized at 121°C for 15 min in an autoclave. Thereafter, the flask material was cooled at room temperature and inoculated with 1 ml spore suspensions in the laminar air flow with the help of sterilized pipette. The flasks were then incubated at 30°C for 5 days.

Optimization of process parameters: Various process parameters were optimized for maximal enzyme production as follows: incubation period (24-144 h), incubation temperature (20-50°C), initial pH (4-9), initial moisture content (0, 2.5, 5, 7.5, 10, 12.5, 15%), inoculum size (2.5, 5, 7.5, 10, 12.5, 15%). Experiments were also performed to evaluate the influence of different carbon sources (maltose, glucose, galactose, lactose, fructose, soluble starch) and nitrogen sources (yeast extract, peptone, tryptone, beef extract, $(NH_4)_3PO_4$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl and $NaNO_3$) on α-amylase production under the optimized fermentation conditions.

Recovery of enzyme: After the specified incubation period (in each case), 50 ml of citrate buffer (pH 5.0) was added in each flask containing fermented matter and placed on a shaker at 200 rpm for 60 min. Afterward, the mixture was filtered and centrifuged at 8,000 rpm for 15 min at 4°C and used as crude enzyme to measure α-amylase activity.

Alpha amylase activity: Enzyme activity was measured by using modified procedure based on method of Okolo *et al*. (1995). The reaction mixture containing 1 ml enzyme extract and 1 ml soluble starch solution (1%) was incubated for 30 min at 50°C. The liberated reducing sugars were estimated by dinitrosalicylic acid (DNS) method of Miller (1959) taking maltose as standard. The blank contained 1 ml distilled water instead of 1 ml enzyme extract. One unit (IU) of α-amylase is defined as the amount of enzyme that liberates 1 µg of reducing sugar as maltose per minute under the standard assay conditions.

Estimation of soluble protein: A method of Lowry *et al*. (1951) was used to measure the soluble protein in the aqueous extract of fermented matter using Bovine Serum Albumin (BSA) as standard.

Statistical analysis: All the data obtained from experiments were statistically analyzed by SPSS software. ANOVA test was applied at significance level of $p < 0.05$

RESULTS AND DISCUSSION

In the present study, α-amylase was produced from *Aspergillus niger* by solid-state fermentation using food waste as substrate. Different process parameters were optimized and characterization of crude α-amylase was carried out.

Chemical analysis of potato peel: Composition of experimental sample of raw potato peel was analyzed and found to contain about 83.5% moisture content. Potato peels also contain approximately 5.68% ash, 2.94% protein and 6.74% fat on dry weight basis. United state department of agriculture (2008) reported that potato peel contained (g/100g of raw skin) water 83.29, protein 2.57, ash 1.61, total fat 0.1 and total dietary fiber of 2.5. Our findings were very similar to that of USDA report and variation in results might be due to difference in environmental conditions.

Effect of incubation period: The rate of production of α-amylase by *Aspergillus niger* in solid-state fermentation is shown in fig 1. Enzyme production started after 24 h of inoculation and increased with the increase in incubation time up to 72 h, reaching its maximum i.e. 1142.70 ± 1.25 U/g and afterward, there was a decrease in amylase production. This might be due to the depletion of nutrients in the medium (Haq *et al*., 2002; Erdal and Taskin, 2010), denaturation of the enzyme (Sindhu *et al*., 2009) or due to production of other by-products (Haq *et al*., 2002). Alpha amylase yield was maximum at 72 h of incubation (Ahmed *et al.,* 2015; Haq *et al*., 2002; Ramachandran *et al*., 2004; Tiwari *et al*., 2007).

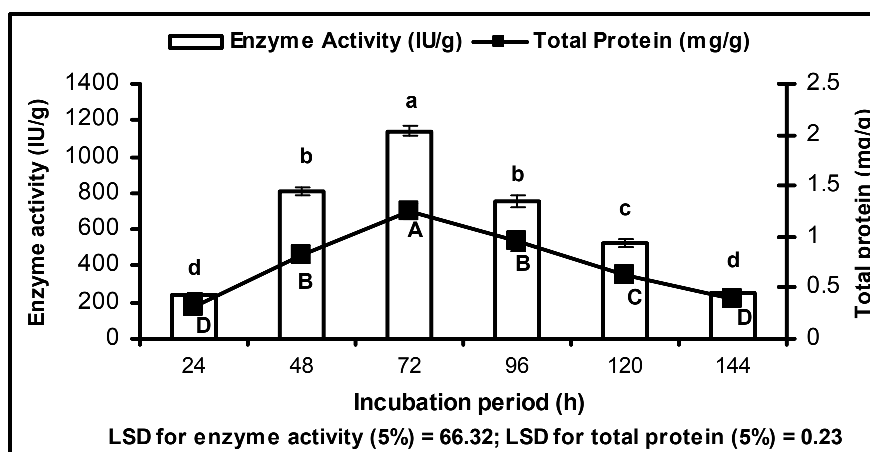

Figure -1: Effect of incubation period on α-amylase and total protein production by *Aspergillus niger*. Standard error and LSD values are shown. (Temperature 30°C, pH 5.0)

Effect of incubation temperature: In the present study, incubation temperature was varied from 20 to 50°C to check the maximum α-amylase production from *A. niger* in solid state fermentation. Results (Fig 2) revealed that maximum α-amylase production was obtained at 30°C (1162.98 ± 1. 34U/g). It was observed that temperature above 45°C resulted in the loss of moisture from substrate, which affects metabolic activities of the microorganism, resulted in reduced growth and enzyme production (Sindhu *et al*., 2009). Previously, 30°C was reported as optimum temperature for amylase production (Irfan *et al.,* 2012; Ramachandran *et al*., 2004; Kunamneni *et al*., 2005; Kathiresan and Manivannan, 2006; Gupta *et al*., 2008; Chimata *et al*., 2010; Erdal and Taskin, 2010; Negi and Banerjee, 2010). Some studies reported that incubation temperature of 35°C yielded best amylase production by *Aspergilus* species indicating little thermophilic nature of strain (Ahmed *et al.,* 2015; Singh *et al.,* 2014).

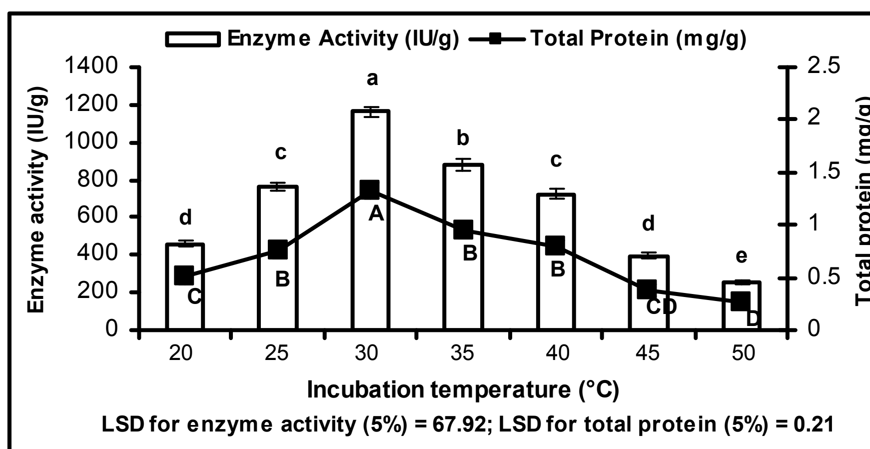

Figure- 2: Effect of incubation temperature on α-amylase and total protein production by *Aspergillus niger*. Standard error and LSD values are shown. (Incubation period 72 h, pH 5)

Effect of initial pH: Effect of initial pH (4-9) of culture medium was determined for best α-amylase production. Maximum α-amylase production (1262.27 ± 2.18 U/g) was obtained at initial medium pH 5 (Fig 3). Below and above this pH, the production of enzyme was lower. Previous studies also reported similar results for α-amylase production at optimum pH 5 (Sivaramakrishnan *et al*., 2007; Gupta *et al*., 2008; Sindhu *et al*., 2009; Chimata *et al*., 2010; Erdal and Taskin, 2010 ; Negi and Banerjee, 2010; Irfan *et al.,* 2012). *Aspergillus fumigatus* produced maximum amylase at initial medium pH of 5.5 (Ahmed *et al.,* 2015). Some strains of Aspergillus niger exhibited initial medium pH of 6.0 for maximum amylase production (Saleem and Ebrahim, 2014).

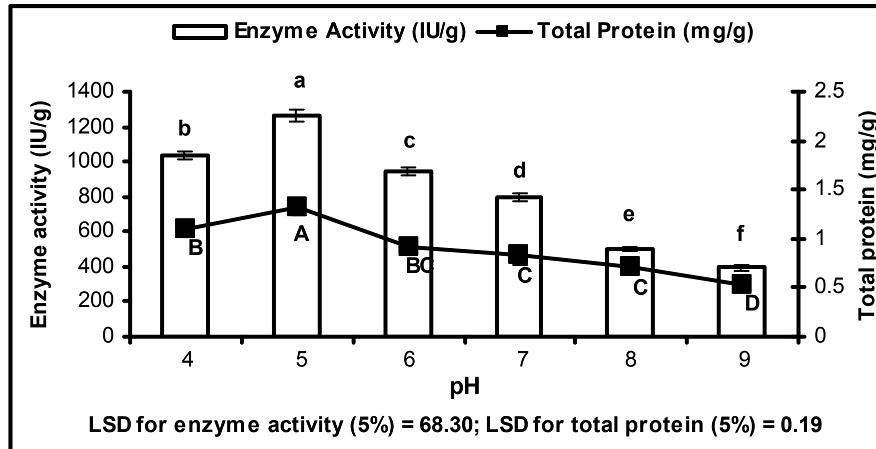

Figure -3: Effect of initial pH on α-amylase and total protein production by *Aspergillus niger*. Standard error and LSD values are shown. (Incubation period 72h, Temperature 30°C)

Effect of moisture level: Effect of various moisture levels (0-15%) on α-amylase production by *Aspergillus niger* is presented in fig 4. Maximum enzyme production (1051.05 ± 1.83 U/g) was observed at 5% moisture level, upon further increase to 15%, there was a gradual decline in enzyme production. Maximum production of amylase was achieved at 70-90% of moisture content as revealed by many researchers (Ramachandran *et al*., 2004; Kunamneni *et al*., 2005; Chimata *et al*., 2010; Erdal and Taskin, 2010 and Negi and Banerjee, 2010). Higher moisture level decreases substrate porosity, promotes stickiness and clumps of substrate, decreases substrate degradation, reduces gas volume and exchange, whereas, lower moisture content may lead to poor solubility and accessibility of nutrients, a lower degree of substrate swelling and higher water tension, resulting in poor microbial growth and decreased enzyme production (Mahanta *et al*., 2008; Zambare, 2010).

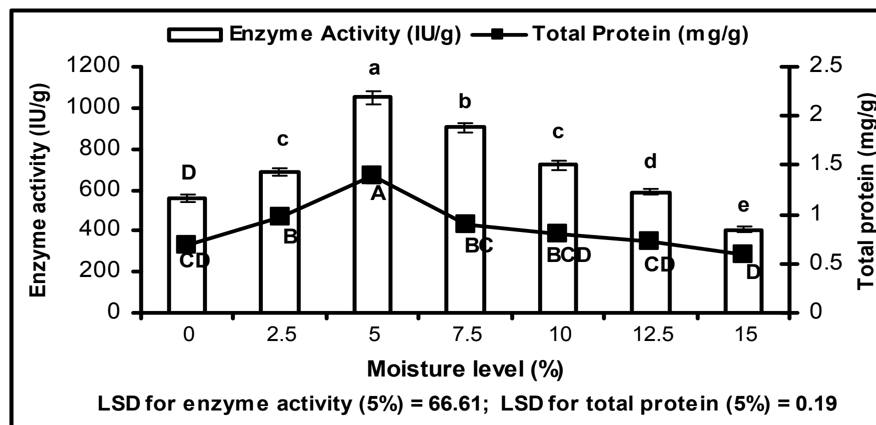

Figure -4: Effect of moisture level on α-amylase and total protein production by *Aspergillus niger*. Standard error and LSD values are shown. (Incubation time 72 h, Temperature 30°C, pH 5.0)

Effect of inoculum size: Figure 5 shows the effect of various inoculum sizes (2.5-15%) on α-amylase production by *Aspergillus niger* in SSF. Highest amylase production (1047.68 ± 1.15 U/g) was attained with 5% inoculum level and then a marginal fall in enzyme production was observed at 15% inoculum size. Similar findings were reported by Kareem *et al.*, (2009) and Chimata *et al.*, (2010) obtained maximum α-amylase yield with 5% inoculum level. Some researchers achieved highest α-amylase production using 10% inoculum level (Ramachandran *et al.*, 2004; Kunamneni *et al.*, 2005). A lower level of inoculum may not be sufficient for initiating growth of fungus and enzyme production. An increase in inoculum size ensures a rapid propagation of biomass and enzyme synthesis. After a certain limit, enzyme production decreased because of depletion of nutrients due to the increased biomass, which resulted in a decreased metabolic activity (Kashyap *et al*, 2002).

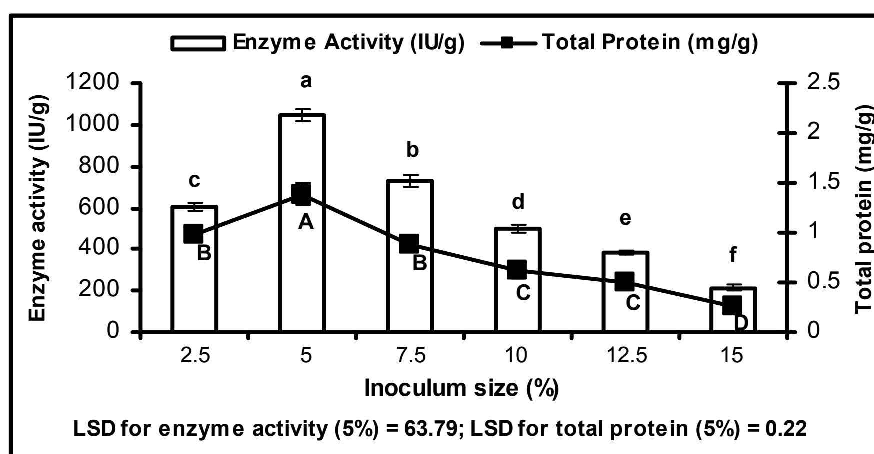

Figure -5: Effect of inoculum size on α-amylase and total protein production by *Aspergilus niger*. Standard error and LSD values are shown. (Incubation time 72 h, Temperature 30°C, pH 5)

Effect of carbon sources: Various carbon sources such as glucose, starch, galactose, lactose, fructose and maltose were tested for maximum α-amylase production by *A. niger* in SSF. Supplementation of the culture medium with different carbon sources for α-amylase production showed increased production of the enzyme (1132.40 ±1.75 U/g) with soluble starch (Fig 6). Similar results were reported by many researchers depicting soluble starch as a best carbon source for alpha amylase production (Ramachandran *et al.*, 2004; Kunamneni *et al.*, 2005; Varalakshmi, *et al.*, 2009; Erdal and Taskin, 2010 and Chimata *et al.*, 2010).

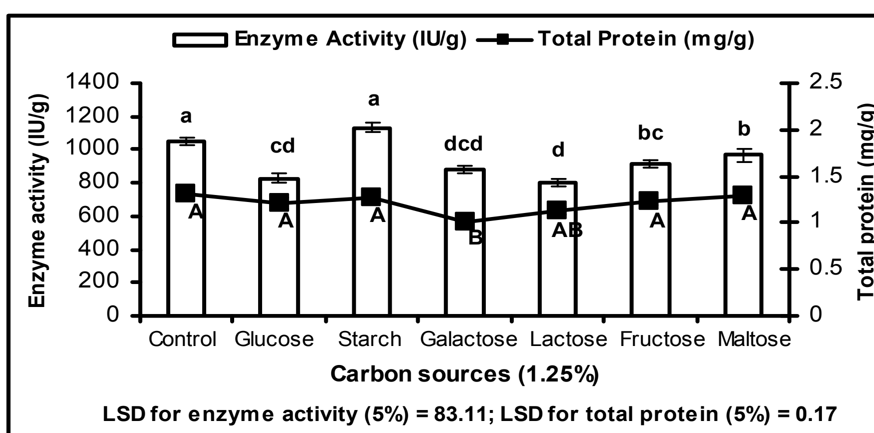

Figure -6: Effect of carbon sources on α-amylase and total protein production by *Aspergillus niger*. Standard error and LSD values are shown. (Control = without external source of carbon) and Incubation period 72 h, Temperature 30°C, pH 5.

Effect of nitrogen sources: Various organic and inorganic nitrogen sources were utilized as supplement to the culture medium for growth of *Aspergillus niger* and production of α-amylase. Results showed that the maximum enzyme production was obtained with peptone (1180.64 ± 1.43 U/g) and ammonium nitrate (1298.12 ± 1.94 U/g) as organic and inorganic nitrogen source respectively (fig 7 and 8). Tryptone, yeast extract and beef extract resulted decreased enzyme production as compared to control. Similar results had earlier been reported in various studies (Ramachandran *et al*., 2004; Kunamneni *et al*., 2005; Kathiresan and Manivannan, 2006; Tiwari *et al*., 2007; Gupta *et al*., 2008; Chimata *et al*., 2010; Erdal and Taskin, 2010; Negi and Banerjee, 2010). Yeast extract was also best nitrogen source for maximum amylase production by *Aspergillus* species (Ahmed *et al.,* 2015; 2014).

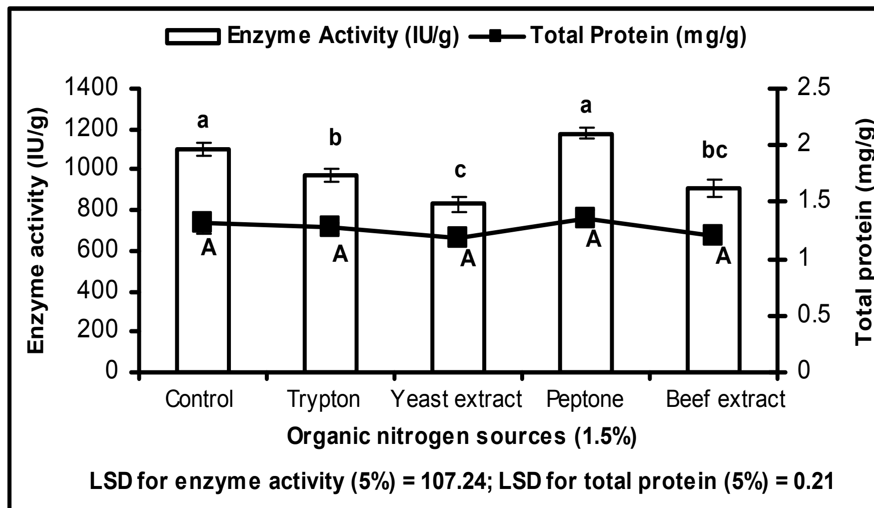

Figure- 7: Effect of organic nitrogen sources on α-amylase and total protein production by *Aspergillus niger*. Standard error and LSD values are shown. Control = without external source of nitrogen when Incubation for 72 h, at Temperature 30°C and pH 5.

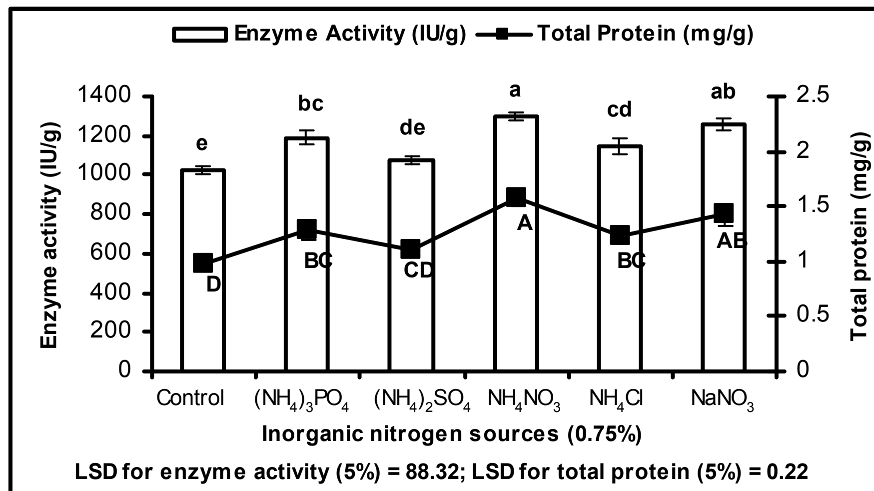

Figure -8: Effect of inorganic nitrogen source on α-amylase and total protein production by *Aspergillus niger*. Standard error and LSD values are shown. Control = without external source of nitrogen when incubation for 72 h at temperature 30°C and pH 5.

CONCLUSION

Results of this study showed that optimization of process parameters were essential for maximum titer of α-amylase production in solid state state fermentation using potato peel as main carbon source. The optimized conditions for maximum amylase (1262.27 ± 2.11 U/g)

production was recorded after 72 h of incubation at 30°C temperature, pH 5 with 5% moisture level and inoculum size of 5% using peptone (1.5%), NH_4NO_3 (0.75%) and soluble starch (1.25%) as nitrogen and carbon source respectively.

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