



Optimization of Protease Production by *Bacillus isronensis* Strain KD3 Isolated from Dairy Industry Effluent

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ABSTRACT

Proteases have a broad range of applications in pharmaceuticals, detergents and food processing industries. Protease producing strains are used profusely in industrial applications and the bioremediation process of wastewaters. In the present research work, efficient protease producing strain was isolated from dairy industry effluent. Screening of protease activity by isolates was checked by growing them on milk agar (skimmed) by spot inoculation method and further estimation was performed using quantitative protease assay. The efficient protease producing strain was identified based on morphological as well as biochemical characteristics as per standard keys of Bergey's Manual of Determinative Bacteriology, later confirmed by 16s rRNA sequencing and BLAST analysis as *Bacillus isronensis* strain KD3. The maximum protease was produced at 42°C; pH 7-8; 200 rpm; and 7% inoculum concentration after 48h of the incubation period.

INTRODUCTION

Enzymes are the biocatalysts, which catalyse multiple biochemical reactions within a short time. Proteases are hydrolases (E.C.3.4.21.14) which breakdown the peptide bonds of amino acids of a protein (Sathyavathan & Kavitha 2013). Proteases have wide applications in pharmaceutical, detergent and food processing industries (Gupta et al. 2005, Verma et al. 2011). Proteases can be defined as enzymes degrading substrate, catalysing complete hydrolysis of proteins (Baroudi et al. 2000). Casein is a major protein occupying 80% of total milk protein. It is a phosphoprotein and different forms of casein depend on the number of phosphate groups attached (Caprita 2008). There are different sources found in nature for protease production like market waste, dairy industrial waste and sewage wastewater (Vasanth & Subramanian 2012). Kolhe & Pawar (2011) reported that the dairy industry effluent has a large amount of protein (especially casein), which may be a good source for isolation of proteolytic bacteria. Patil & Kurhekar (2018a) has reported that bacterial protease has more significance in the industry as compared to animal and fungal proteases. Appleby (1955) isolated proteolytic bacteria from rumen of sheep. Kamoun et al. (2008) have reported some protease producing bacterial strains namely *Bacillus subtilis*, *Bacillus alcalo*, *Bacillus licheniformis*, *Bacillus thuringiensis*, *Bacillus firmus* and fungal strains such as *Aspergillus miller*, *Penicillium griseofulvin*, *Aspergillus flavus* and *Aspergillus*

niger. Patil & Kurhekar (2018b) screened caseinase producing bacteria from dairy industry effluent and further studied them quantitatively.

Commercially available proteases are stable at variable pH and are highly active (Maurer 2004). Mostly produced by different *Bacillus* species, especially alkaline proteases have wide substrate specificity and purification cost is very low (Haddar et al. 2010). Protease producing strains have a large range of applications in food, detergent, textile, tanning, leather and pharmaceutical industries (Prakasham et al. 2005). To check the effect of physiological parameters on production of protease, various factors like medium pH, temperature, agitation speed, inoculum concentration, incubation time and nutrient supplements like sources of carbon as well as nitrogen were taken into the consideration (Badhe et al. 2016). The present investigation aimed to screen strain producing protease from dairy industry effluent and to optimize protease production for different cultural conditions.

MATERIALS AND METHODS

Dairy Industry Effluent

The dairy industrial effluent was obtained from the Effluent Treatment Plant (ETP) of a dairy industry located in Pune district, Maharashtra, India. The effluent samples for analysis were collected and kept in clean and sterile glass containers,

stored in an icebox at low temperature and immediately transported to the laboratory, for further analysis. Before use, the effluent samples were maintained at low temperature, i.e. 4°C in a refrigerator to avoid any physicochemical change (APHA 2005, Trivedy & Goel 1984).

Isolation and Screening of Protease Producing Bacteria

The protease producing bacteria were isolated from dairy industry effluent by the enrichment method. It was performed in a 250 mL of Erlenmeyer flask with 100 mL sterile nutrient broth, inoculated with 10 mL of effluent sample. The flasks were placed overnight on a rotary shaker at 150 rpm at 37°C. A loop full of enriched culture streaked on sterile nutrient agar plates was incubated at 37°C for 24 h. Morphologically different isolated colonies were streaked on nutrient agar plates, for obtaining pure cultures. Cultural characteristics of isolates were studied. Nutrient agar slants were used for maintaining pure cultures at 4°C. The screening was done to find out the most potential bacterial isolate with proteolytic activity. For this study, colonies with different morphologies were marked for future study. Protease activity was qualitatively detected using Skimmed Milk Agar (Skimmed Milk Powder - 10 %, Peptone - 0.5 %, Agar Agar - 2.5%, pH - 7.2). The halo casein hydrolysis zone was seen on the plate after the incubation at 30°C for 24 and 48 h. The zone diameter for each isolate was measured after 24 hours of the incubation period. The strain with maximum protease activity (qualitatively) was used for further study.

Protease Assay

The protease enzyme assay was performed with some modifications in the protocol suggested by Tsuchida et al. (1986) involving casein as substrate. The bacterial isolate was grown in Nutrient Broth with 1% casein as a substrate and then incubated for 24 h at 30°C. Cell-free supernatant was subjected to enzyme assay. Enzyme solution, i.e. supernatant (100 µL) was added to 900 µL substrate (2 mg/mL (w/v) solution of casein in Tris-HCl buffer (10 mM) with pH 8.0. Incubation of the mixture was carried out for 30 min at 45°C. The reaction was stopped with the addition of chilled trichloroacetic acid 10 (w/v) in equal volume and kept for 15 min, which allowed the insoluble proteins to precipitate. By centrifugation for 10 min, at 10000 rpm at 4°C, neutralization of supernatant was carried out with 5 mL 0.5 M Na₂CO₃ solution (0.5 M). With the addition of 3-fold diluted Folin-Ciocalteu reagent, optical density was measured at 600 nm in triplicate. One protease unit can be defined as the enzyme amount that produces 1 µg tyrosine per mL per min, under standard assay conditions (Lowry et al. 1951).

Identification of Protease Producing Isolate

Bacterial isolate exhibiting highest zone of protein hydrolysis was used further for study. The efficient protease producing strain was identified based on morphological as well as biochemical characteristics as per standard keys of Bergey's Manual of Determinative Bacteriology (Bergey et al. 1974), and later confirmed by 16s rRNA sequencing and BLAST analysis.

Optimization of Protease Production for Different Cultural Conditions

For optimization of protease production at different pH, the bacterial isolate was grown separately in Nutrient Broth supplemented with 1% casein and pH of 4, 5, 6, 7, 8, 9, 10 and 11 at 37°C. To optimize the temperature conditions, the temperatures were set as 20, 25, 30, 37, 42 and 50°C. The highest caseinase production was recorded in a quantitative mode and the same was continued for further study. To investigate the impact of different speeds of agitation, the efficient bacterial isolates were allowed to grow separately in Nutrient Broth supplemented with 1% casein at variable shaking conditions of 100, 150, 200, 250, 300 and 350 rpm at optimum pH and temperature value recorded earlier. Inoculum concentration of 1% to 10% was tested separately in Nutrient Broth medium having 1% casein with optimum conditions as per the earlier study. To observe the impact of incubation time, the isolate was tested separately in Nutrient Broth medium supplemented with 1% casein with all the optimum conditions and tubes were incubated separately for the period of 8, 16, 24, 32, 40, 48, 56, 64 and 72 h. Further, the suitable incubation time was recorded based on protease assay and continued for further study. Carbon sources were tested as 1%, i.e. maltose, glucose, starch and sucrose separately. Different nitrogen sources were screened for 1% ammonium sulphate, tryptone, beef extract and malt extract. The physicochemical parameters were selected as per the earlier study. The production of protease was determined by quantitative assay.

Statistical Analysis

The experimental data were analysed using Bonferroni's multiple comparison test.

RESULTS AND DISCUSSION

Isolation and Screening of Protease Producing Bacteria

Thirty morphologically distinct bacterial colonies were isolated on Nutrient Agar medium from selective enrichment of dairy industry effluent samples. To screen protease producing

strains; individual culture of each isolate was spot inoculated in centre of Milk Agar plates. Positive strains were evaluated by observation of clear hydrolysis zone surrounding the colony. In all, twelve bacterial strains namely KD1, KD2, KD3, KD4, KD5, KD6, KD7, KD8, KD9, KD10, KD11 and KD12 (Assigned names for laboratory use only) were identified as protease producers due to their clear zone of casein hydrolysis around the colony. The casein hydrolysis zone was measured for each isolate and results are given in Table 1. As per qualitative test results isolate KD3 showed high protease activity as compared to remaining isolates (Fig. 1). So, isolate KD3 was used for further study.

Phylogenetic Identification of Selected Bacterial Strain

The selected bacterial strain with higher protease activity was identified as *Bacillus isronensis* strain *KD3* based on morphological as well as biochemical characteristics as per

standard keys of Bergey's Manual of Determinative Bacteriology (Bergey et al. 1974) and by molecular characterization (16s rRNA sequencing) (Fig. 2). The 16s rRNA sequence of *Bacillus isronensis* strain *KD3* was compared with NCBI-BLAST data bank and then deposited in NCBI data bank (Accession No. LC260011) for the phylogenetic analysis. DND file obtained from CLUSTAL alignment was employed for phylogram built up by using the MEGA5 software. The built phylogram was documented with close homology of the bacteria isolated with the best matched bacterial sequence and highlighted by marking in a phylogram (Fig. 3).

Optimization of Protease Production for Different Cultural Conditions

Effect of pH on the production of protease: pH strongly affects ion transport and nutrients across the cellular membrane. It also plays an important role in multiple enzymatic

Table 1: Diameter of clear hydrolysis zone surrounding the bacterial isolates on Milk Agar plates for 24 hours at 30°C.

Bacterial isolates	Diameter of zone surrounding the colony (mm)	Protease activity (U/mL)
KD1	7	50
KD2	10	81.5
KD3	18	120.4
KD4	12	94.5
KD5	13	96.8
KD6	8	54.2
KD7	7	50
KD8	14	101
KD9	15	107
KD10	12	95
KD11	11	85
KD12	10	81.5

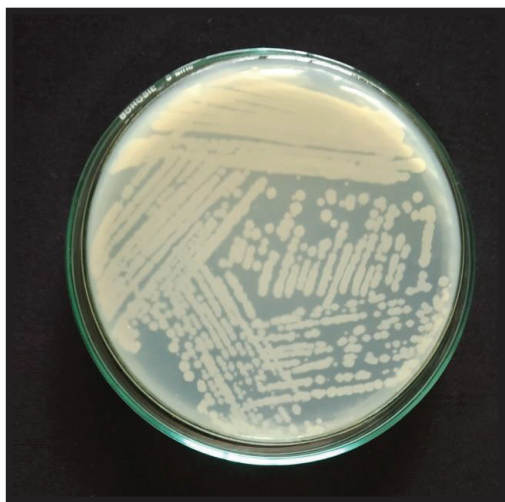


Fig. 1: *Bacillus isronensis* strain *KD3* on the nutrient agar plate.

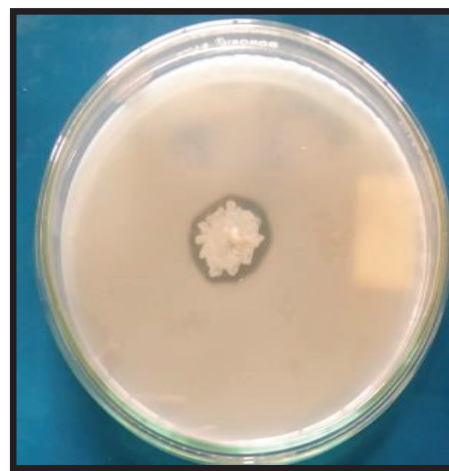


Fig. 2: *Bacillus isronensis* strain *KD3* on milk agar showing zone of proteolysis.

processes (Moon & Parulekar 1991). In the present study, production of protease was observed as negligible in isolate, set at pH 4, 5, 10 and 11 while best protease production was noted at pH 7 and pH 8 (Fig. 4). *Bacillus isronensis* strain KD3 was preferred as it produced higher protease ($P < 0.0001$) at alkaline pH with 41 ± 0.51 U/mL. The production was observed to prefer neutral to mildly alkaline conditions for protease production. In a related study, Kembhavin et al. (1993) reported that thermostable alkaline protease, isolated from *Bacillus subtilis* NCIM No. 64, was capable of growing in a medium set at varying pH of 5-11 and in all these conditions it was able to produce protease successfully. According to Nascimento et al. (2004), thermophile *Bacillus* sp. was able to produce optimum protease activity at pH 8 and it was reduced when pH was set at 5.5 and 9, after incubating crude

enzyme for 24 h. In another report, alkalophilic *Bacillus* sp. JB 99 was able to produce protease activity at pH 11 with the temperature set at 70°C and confirmed to be serine alkaline protease in feature (Johnvesly & Naik 2001).

Effect of temperature on production of protease: Induction of protease production in the medium inoculated with isolate was allowed to manifest by providing incubation at different temperatures ranging 20, 25, 30, 37, 42 and 50°C sets. *Bacillus isronensis* strain KD3 was reported with maximum production at temperature 42°C with 59 ± 0.81 U/mL value and later on with further increase or decrease in temperature, a sharp fall in activity was recorded (Fig. 5). According to Nascimento et al. (2004) protease activity in a thermophilic *Bacillus subtilis* grown in liquid medium

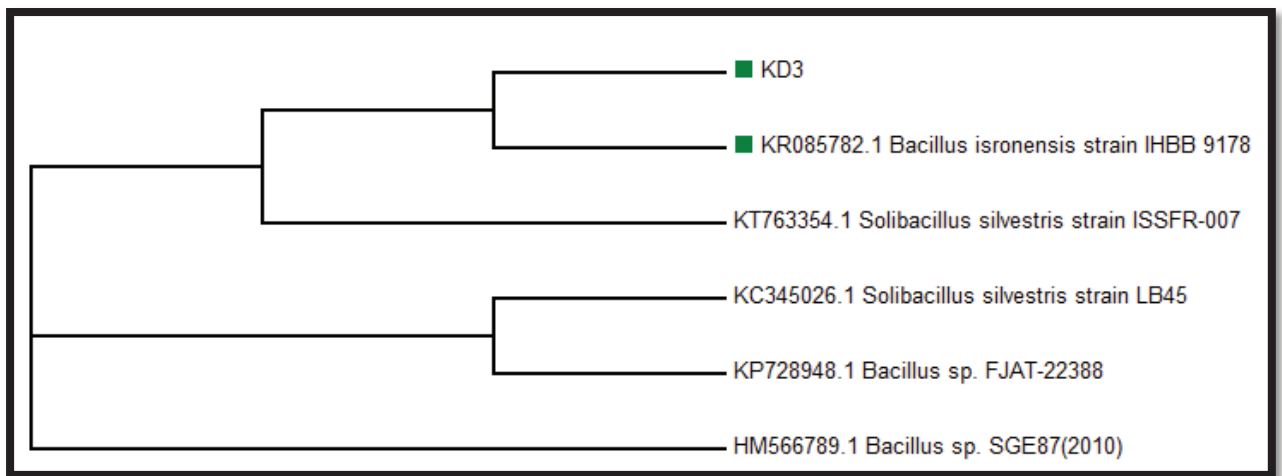


Fig. 3: Phylogram of *Bacillus isronensis* strain KD3.

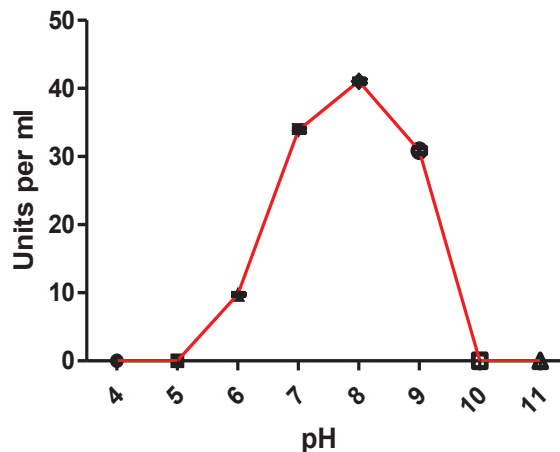


Fig. 4: Effect of pH on the production of protease.

supplemented with trisodium citrate was able to produce optimum protease at 60°C since the bacterium was a thermophile. In the case of our study, we deduced that protease production remained at its best with the temperature of 37°C-42°C considering that our isolates are mesophilic. In a similar study and report, which was very close to our findings, Kanekar et al. (2002) showed that isolates of genera *Bacillus*, *Staphylococcus*, *Micrococcus*, *Pseudomonas* and *Arthrobacter* exhibit optimum protease activity when the medium was maintained at 30°C under shaking conditions. Kumar (1999) reported the production of protease by *Bacillus* species was 84.09 U/mL at 45°C.

Effect of speed of agitation on the production of protease:

In the present investigation, protease production was shown to be affected by agitation speeds applied to the medium. Agitation speed was set at 100-350 rpm, recorded that between 150 and 250 rpm it was able to produce better protease in all media (Fig. 6). *Bacillus isronensis* strain *KD3* showed maximum protease production at 200 rpm with 49 ± 0.058 U/mL and second best was shown at 150 rpm (26 ± 0.32 U/mL). As the revolution increased, activity lowered down speedily and reached to the minimum of 8.8 ± 0.26 U/mL at 350 rpm. As per the results, agitation speed set at 150-200 rpm was most optimum for protease production in the study. As per Escobar & Barnett (1993) enzyme production remained in direct proportion to shaker speed when they investigated the recovery of mould acid protease from *Mucor miehei* CBS 370.65. Genckal & Tari (2006) reported while studying *Bacillus* sp. strain *I18*, *L18*, and *L21* that better protease and growth could be achieved when conditions were set at 30°C (strain *I18*) and 37°C for *L18* and *L21* strains. Similarly, agitation speed was 100 rpm for *I18* and 180 rpm for *L18*

and *L21*. This study is similar to our finding, where the use of 150-200 rpm is the normal range reported.

Effect of inoculum concentrations on production of protease: Optimization of protease production was carried out for different inoculum concentration set as 1% to 10%. *Bacillus isronensis* strain *KD3* reported better production with 6% with 39 ± 0.68 U/mL and showed improved activity for 7% inoculum concentration which gives 42 ± 0.3 U/mL of activity (Fig. 7). Later on, a decrease in the value was recorded. A significant difference from 1% to 10% bacterial inoculation of protease production showed better activity with 7% inoculation significantly ($p < 0.05$). In the present investigation, the effect of concentration of inoculum on the production of protease has been put forth. It was noted that inoculum in the range of 5-7% improved caseinase production at its best as compared to 1-3% and 8-10% concentration.

Impact of time of incubation on the production of protease: *Bacillus isronensis* strain *KD3* was reported to be better protease producer at 48 h (42 ± 0.56 U/mL) (Fig. 8). It was negligible during the first 16 h and decreased beyond 56 h till 80 h. That was the incubation time last recorded. As observed, the highest production of protease was obtained at 48-56 h of incubation by the isolates. In a similar study, Beg et al. (2003) reported that in a shake flask culture *Bacillus mojavensis* was able to produce protease within 24 h with an activity of 558 U/mL and it was further reduced down in a bioreactor to 10-12 h. In a similar study, production of alkaline protease in *Bacillus* species was investigated in the fermentation of solid-state type and increased protease activity was reported at 24 h when lentil husk and wheat bran were used as substrate (Uyar & Baysal 2004).

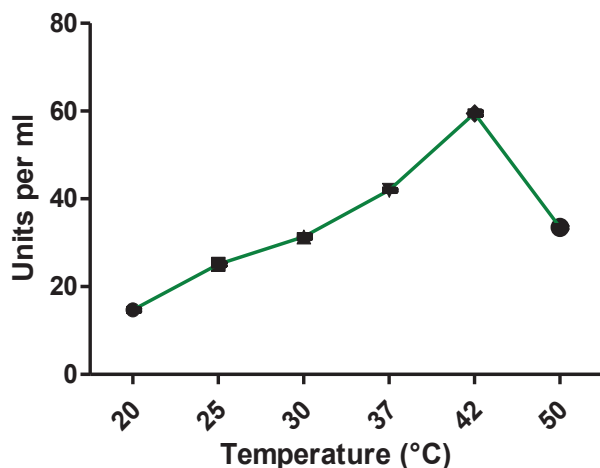


Fig. 5: Effect of temperature on the production of protease.

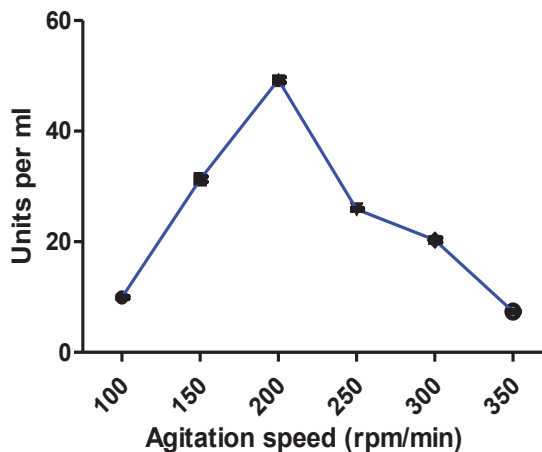


Fig. 6: Effect of speeds of agitation on the production of protease.

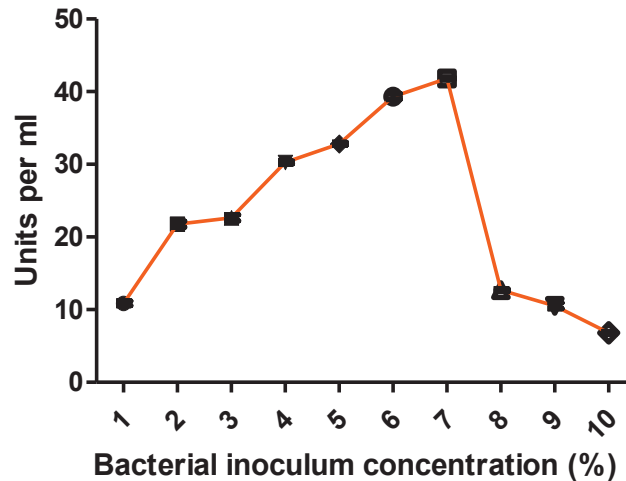


Fig. 7: Effect of inoculum concentration on the production of protease.

Effect of various sources of nitrogen on the production of protease: Among the all nitrogen sources (1%) tested, production of protease was observed to get affected by the supplement such as ammonium sulphate, tryptone, beef extract and malt extract (Fig. 9). As compared to control, *Bacillus isronensis* strain *KD3* not get induced by any nitrogen source and value of protease production was reported to be either low or constant to control. This indicated a neutral effect of source of nitrogen on protease production. Use of ammonium sulphate was found improving protease production as reported by Varela et al. (1996) working with *Bacillus subtilis*. Rahman et al. (2005) also strongly supported the finding that strain *K* of *Pseudomonas aeruginosa* was able to produce stable protease when nitrogen source as tryptone, soybean and yeast extract was provided.

Effect of various sources of carbon on the production of protease: During the carbon source optimization study, *Bacillus isronensis* strain *KD3* was not affected by the carbon source addition as no stable increase in protease was observed in any set of carbon source (Fig. 10). In the present study, carbon source glucose was reported better for protease activity. In a similar study, glucose with a concentration of 2 mg/mL was reported as the optimum concentration for production of protease in *Bacillus mojavensis* when studied in 14 L bioreactor, as predicted by the statistical model. It was also reported that >2 mg/mL level of glucose turned out to be responsible for catabolic repression in protease production (Beg et al. 2003). Kole et al. (1988) advocated for simultaneous control of ammonium and glucose concentrations in *Bacillus subtilis* in fermentation conditions for better protease

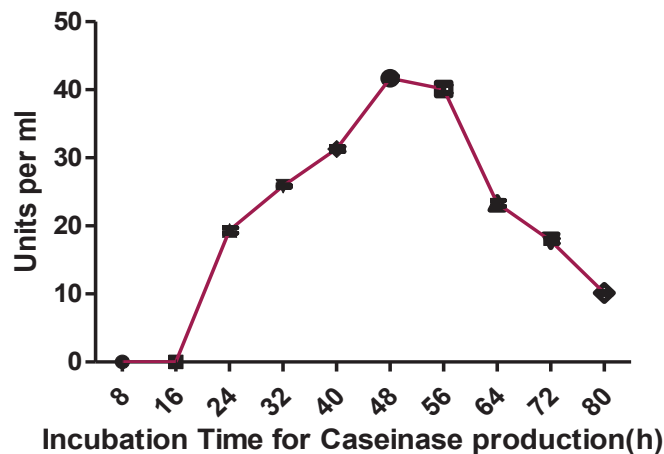


Fig. 8: Impact of incubation time on the production of protease.

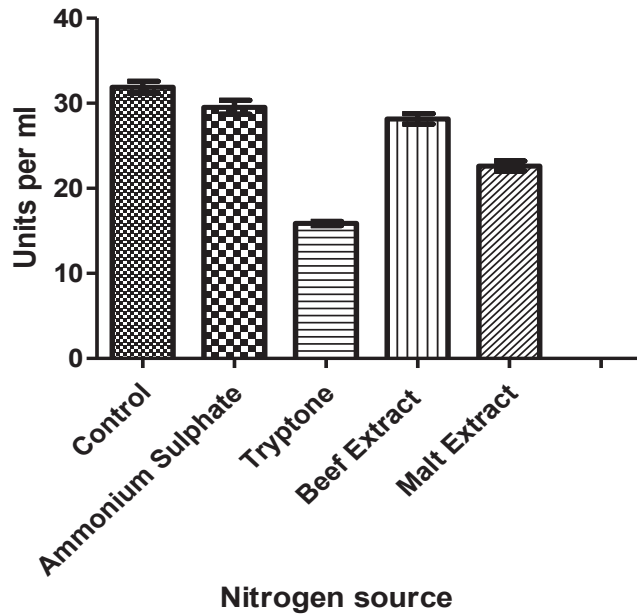


Fig. 9: Effect of sources of nitrogen on the production of protease.

production. In a report, glucose supplemented in a medium with a pH range of 9.5-11.5 and temperature 65°C, was able to induce protease production in *Microbacterium* sp. isolated and identified from Ethiopian alkaline soda lake. This study also supports our finding that glucose acts as a promoter for better protease activity (Gessesse & Gashe 1997). Similarly, starch has been reported positive for better protease production as reported by Naidu & Devi (2005), Reddy et al. (2008) and Feng et al. (2001) while investigating the *Bacillus* sp.; *Bacillus* sp. RKY3 and *Bacillus pumilus* strain respectively.

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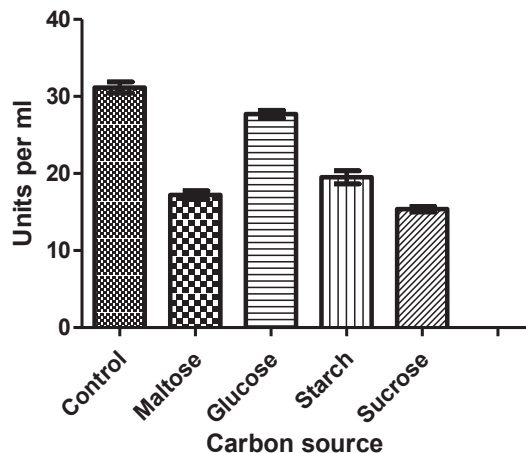


Fig. 10: Effect of various sources of carbon on the production of protease.

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