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ISOLATION OF THERMO ALKALINE PROTEASE PRODUCING BACTERIA AND EVALUATION OF AGRO-WASTE FOR PROTEASE PRODUCTION

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ABSTRACT

In the present study, a potential protease producing isolate designated ASKL-09 was obtained from the garden soil by serially dilution. Based on morphological, microscopic, biochemical and 16S rRNA sequencing the isolate was identified as *Bacillus sonorensis*. The optimum pH and temperature was pH 9 and 50˚C respectively at 30 h. Carbon source (glucose at 1.5 %) and nitrogen source (beef extract 0.5%) and metal ion (magnesium sulphate at 0.05%) showed highest protease production. *Bacillus sonorensis* ASKL-09 produces protease at high pH and temperature suggesting it could useful for industrial applications.

KEYWORDS: Alkaline protease; submerged fermentation; agro-wastes; *Bacillus sonorensis*.

1. INTRODUCTION

Proteases are complex group of enzymes collectively known as peptidyl-peptide hydrolases and are responsible for the hydrolysis of peptide bonds in protein molecules.[1] Proteases constitute one of the most important groups of enzyme and accounting for nearly 60% market value due to their wide scale applications in the detergents, food, pharmaceuticals, chemicals, leather, paper and pulp and silk industries.[2, 3] A large group of microbes belonging to bacteria, yeast, fungi and actinomycetes are known to produce proteases,[4, 5] but most of these enzyme can`t meet the industrial standards. Further industrial production of these enzymes is feasible only at a competitive price.[1] Hence, in the present study an effort to obtained thermo alkaline protease was made. Further, to reduce the production cost agro-waste were screened for protease production.

2. MATERIALS AND METHODS

2.1. Isolation and screening of alkaline protease producing bacteria

Garden soil samples from Gulbarga University, Karnataka, India were collected during the month of March 2015. The soil samples were serially diluted and 100 µl of the aliquot was spread over the Nutrient agar (NA) containing 1% Skim milk. The plates were incubated for one week at 45˚C. The colonies with zone of hydrolysis were considered positive for alkaline production.

A total of 12 isolates showed zone of hydrolysis, which were selected and designated as ASKL 01-12. The isolates were purified using the same media. Among 12, isolate designated ASKL-09 showed highest zone of clearance which was selected for further work.

2.2. Identification of isolate ASKL-09

The isolate ASKL-09 was identified based on morphological, microscopic, biochemical and 16S rRNA sequencing. Gram staining was performed using the standard Gram reaction. The microscopic properties were observed with the aid of light microscopy. IMVIC test was performed as per the protocol described by Aneja, (2003).[6] Catalase activity was determined based on the production of bubbles after the addition of a drop of 3% (v/v) H_2O_2. Oxidase activity was determined based on oxidation of tetramethyl p-phenylenediamine.[7] Other biochemical characters, such as starch and Tween (40 and 80) hydrolysis were performed as described by Gonzalez *et al.*, (1978).[8]

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene of isolate ASKL-09 was performed as described by Li *et al*., (2007).[9] The obtained sequence was compared with available 16S rRNA gene sequences of cultured species from the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al*., 2012).[10] The obtained sequences were aligned using CLUSTAL_X program (Thompson *et al*., 1997).[11] Phylogenetic tree was constructed using the aligned sequences by the neighbor joining method using Kimura-2-parameter distances in MEGA 5 software.[12-14]

2.3. Screening of agro-wastes for the production of protease using submerged fermentation

Agro wastes such as wheat bran, soya bean, ground nut cake, green gram husk, sunflower seed cake and red gram husk were selected for the production of protease using submerged fermentation. All the substrates were grinded to fine powder. 10 g of each substrate was

dissolved in 100 ml of distilled water taken in 250 ml conical flask. The flasks were autoclaved at 121 °C for 15 min and allowed to cool at room temperature.

The inoculum was prepared using 18 h old culture which was scraped aseptically and transferred to 10 ml sterile distilled water this suspension have approximately 3×10^7 cells/ml. About 100 µl of the suspension was inoculated in each flask and then incubated at 45 °C. The enzyme was assayed at every 6 h.

2.4. Enzyme assay

The enzyme assay was performed as per the standard protocol described by Charles *et al.*, 2008.[15] 1ml of the enzyme was mixed with 1ml of 1% casein solution. The mixture was incubated at 37˚C for 10 min. The reaction mixture was stopped by adding 2ml of 0.4 M TCA. The mixture was again incubated at 37˚C for 20 min. The mixture was centrifuged at 10,000 rpm 10 min at 4˚C. Then 1ml of the supernatant was mixed with 5ml of 0.4 M Na_2CO_3 and 1ml of Folin phenol reagent. The mixture was incubated for 20 min and the optical density was measured at 660 nm. One unit of enzyme is defined as the amount of enzyme required to liberate 1µmol of tyrosine in 20 min at 37 ˚C.

2.5. Optimization of parameters for protease production by isolate ASKL-09

In the present study, parameters like pH, temperature and agitation speed was optimised for the production protease by isolate ASKL-09. The submerged fermentation was carried out using wheat bran as a substrate (10 g in 100 ml). The effect of pH was evaluated at a pH ranging 6-11 (at interval of 1). The pH was adjusted using buffers: acetate buffer (for pH6), phosphate buffer (for pH 6-7.5), Tris Hcl (for pH 7.5-9.0) and carbonate buffer (for 9-11). The effect of temperature was evaluated at a temperature ranging from 30-65˚C (with increment of 5˚C). The effect of agitation was evaluated at 80, 120, 140, 180 and 220 rpm.

2.6. Influence of additional source on protease production by isolate ASKL-09

The influence of additional source such as carbon, nitrogen and metal ions on protease production by isolate ASKL-09 was evaluated. The production broth (10 g of wheat bran in 100 ml) was further supplemented with carbon source such as glucose, sucrose and starch at a concentration ranging 0-2.5% (with increment of 0.5%), nitrogen source such as peptone, beef extract and potassium nitrate at a concentration ranging 0-2.5% (with increment of 0.5%) and metal ions such as ferrous sulphate, zinc sulphate and magnesium sulphate at a concentration ranging 0-0.15% (with increment of 0.05%).

3. RESULT AND DISCUSSION

3.1. Isolation and Identification of potential protease producing isolate ASKL-09

The potential protease producing isolate ASKL-09 was isolated from the garden soil sample of Gulbarga University, Karnataka, India. The isolate shows zone of hydrolysis on NA containing 1% Skim milk (Figure 1). The colony color was cream, irregular shape. The microscopic results revealed that the isolate ASKL-09 was Gram positive, rod shaped (Figure 2) and motile. In biochemical tests, the isolate was indole negative; Methyl red, voges proskauer and citrate utilization positive. A detailed morphological, microscopic and biochemical tests were mentioned in Table 1. Based on the above results the isolate ASKL-09 was preliminary confined to the genus *Bacillus*. The 16 S rRNA sequencing showed that the isolate ASKL-09 maximum similarity (100 %) with *Bacillus sonorensis.* Further, the neighbor joining tree (Figure 3) showed the isolate ASKL-09 clad with *Bacillus sonorensis.* Based on morphological, microscopic, biochemical and 16S rRNA sequencing the isolate was identified *Bacillus sonorensis* ASKL-09.

Table 1: Morphological, microscopic and biochemical characters of isolate the ASKL-09

SL No	Morphological, microscopic and biochemical Characters	Results
1.	Colony color	Cream
2.	Colony Shape	Irregular
3.	Gram Staining	Positive
4.	Shape	Rod shaped
5.	Motility	Motile
6.	Endo spores	Positive
7.	Indole	Negative
8.	Methyl red	Positive
9.	Voges proskauer	Positive
10.	Citrate utilization	Positive
11.	Gelatin hydrolysis	Positive
12.	Catalase	Positive
13.	Oxidase	Negative
14.	H_2S production	Negative
15.	Urease	Positive
16.	Tween 40 And 80	Positive
17.	Starch hydrolysis	Positive

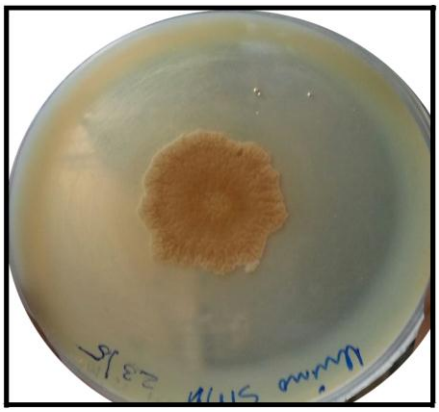

Figure 1: Isolate ASKL-09 showing zone on hydrolysis on nutrient agar containing 1% skim milk.

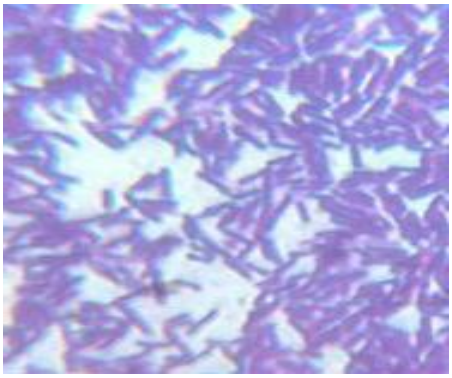

Figure 2: Microscopic characters of isolate ASKL-09 showing Gram positive, rod shaped bacteria.

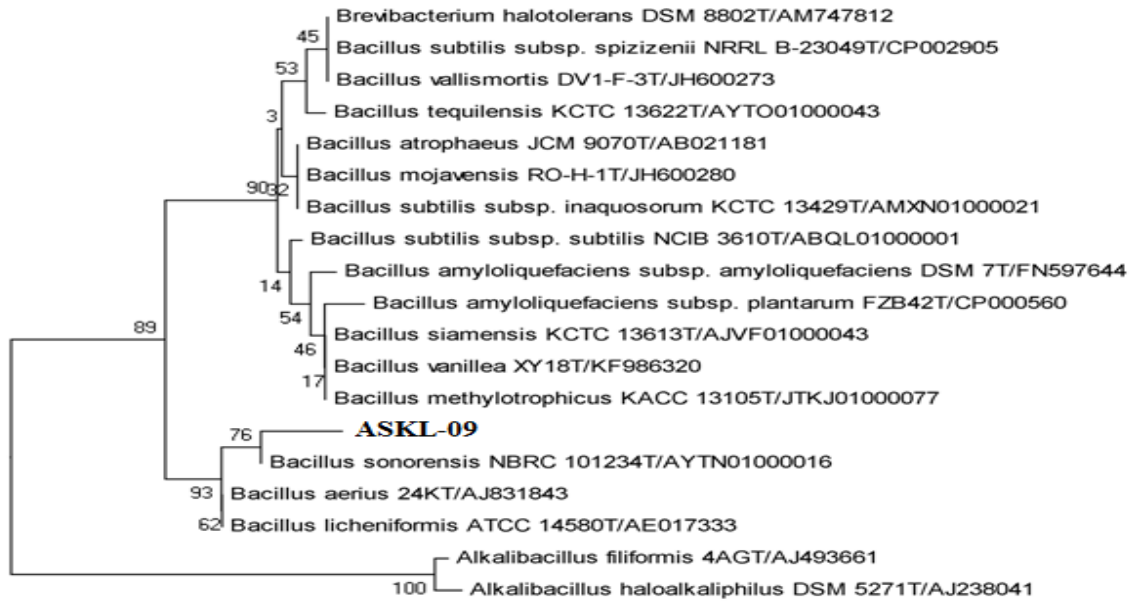

Figure 3: Neighbour joining tree based on 16S rRNA sequencing. Numbers at the node represents bootstrap values.

3.2. Screening of agro-wastes for the production of protease using submerged fermentation

Among various substrate used for the production of protease, *Bacillus sonorensis* ASKL-09 showed highest protease production in wheat bran followed by soya bean, sunflower seed cake, green gram husk, red gram husk and lowest in ground nut cake (Figure 4). In contrast to our study, Sankareswaran *et al.*, (2014) showed maximum protease production in ground nut cake by *Bacillus* sp. indicating the substrate required for protease production varies with different strain[16].

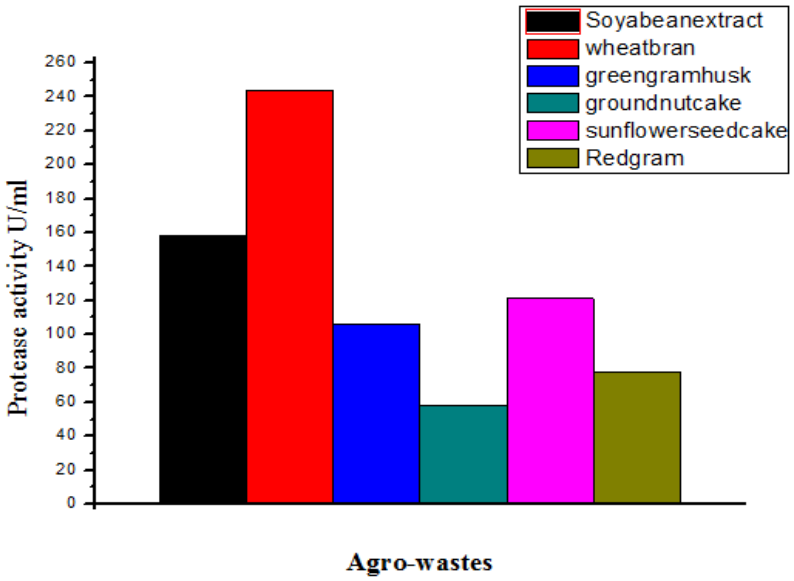

Figure 4: Submerged fermentation of *B. sonorensis* ASKL-09 showing maximum protease production in wheat bran (244 IU/ml).

3.3. Optimization of parameters

The parameters like pH, temperature and agitation speed for protease production by *B. sonorensis* ASKL-09 was evaluated. Figure 5 show that *B. sonorensis* ASKL-09 produce maximum protease at pH 9 (271 IU/ml) at 30 h, while least at pH 6. The result suggests that the protease produced by *B. sonorensis* ASKL-09 is alkali stable. Similarly *B. sonorensis* ASKL-09 produce maximum protease at 50 ºC 284 IU/ml (Figure 6) suggesting thermo stable enzyme production. Further, the maximum protease was noticed at 180 rpm (298 IU/ml) (Figure 7). Similarly, under submerged fermentation conditions, Gaurav *et al.*, 2015 reported a high level of protease production at 45 °C after 36 h at pH 10, with continuous agitation (180 rpm) by *B. subtilis.*[17]

3.4. Influence of additional source on protease production by *B. sonorensis* ASKL-09

Figure 8 depicts the influence of carbon source on protease production by *B. sonorensis* ASKL-09 showing maximum activity (321 IU/ml) at 1.5 % glucose concentration followed by sucrose at concentration 1.5% and starch 0.5 % at 30 h. The result suggests that protease production by *B. sonorensis* was more in simple sugars when compared with complex. Further, *B. sonorensis* ASKL-09 showed highest protease production in organic nitrogen source when compared with inorganic nitrogen source. The maximum protease production was observed in beef extract at 0.5% concentration followed by peptone 0.5% concentration. Lowest production was observed in potassium nitrate (Figure 9). The metal ions greatly influenced the protease production by *B. sonorensis* ASKL-09. Among various metal ions, magnesium sulphate at 0.05% showed highest protease production (315 IU/ ml). All metal ions at concentration higher than 0.05% hinder the production. Within the genus *Bacillus* the maximum protease activity was observed in different carbon and nitrogen sources.

Vanitha *et al.*, (2014) [18] observed maltose as best carbon source while yeast extract as good nitrogen source using wheat bran substrate by *Bacillus subtilis* 168. Gaurav *et al.*, 2015 found maximum production of protease by *Bacillus subtilis* in peptone at concentration 0.5 %. Nurullah Akcan (2012) found Urea and sodium nitrate were the best nitrogen sources for protease production by *Bacillus licheniformis* ATCC 12759 while $FeSO_4$, $ZnSO_4$ and CuSO4 completely repressed protease production. [19] From the above it can be said that different additional source have different activity on protease production.

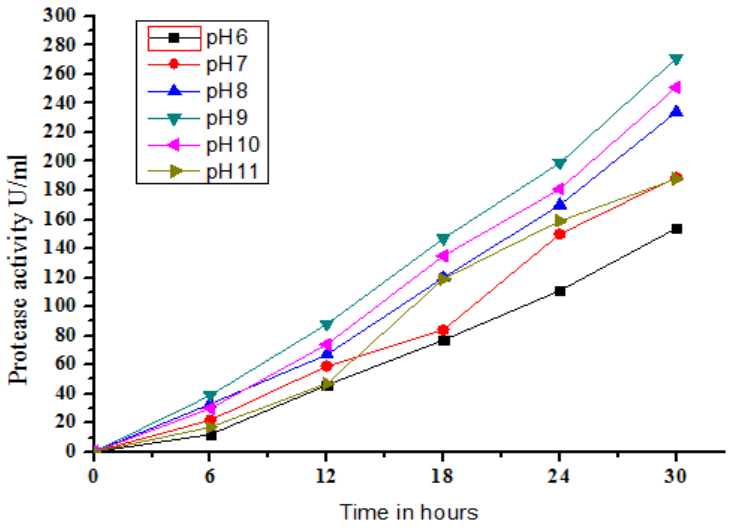

Figure 5: Protease production by *B. sonorensis* ASKL-09 showing maximum activity at pH 9 (271 IU/ml)

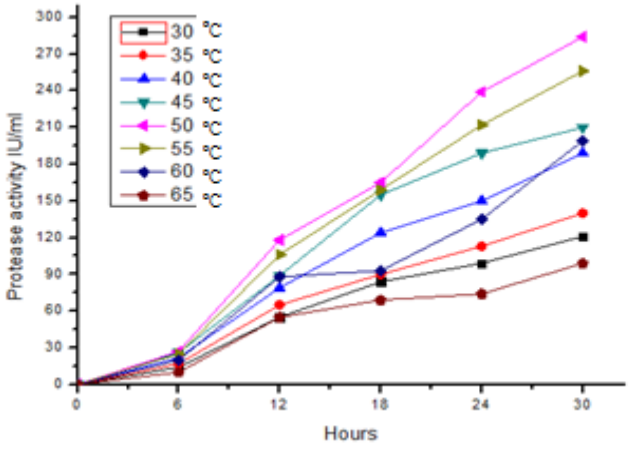

Figure 6: Protease production by *B. sonorensis* ASKL-09 showing maximum activity at 50°C (284 IU/ml).

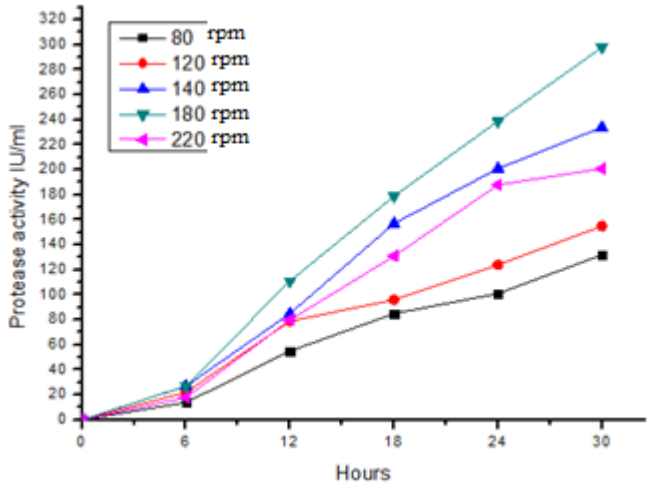

Figure 7: Protease production by *B. sonorensis* ASKL-09 showing maximum activity at 180 rpm (298 IU/ml).

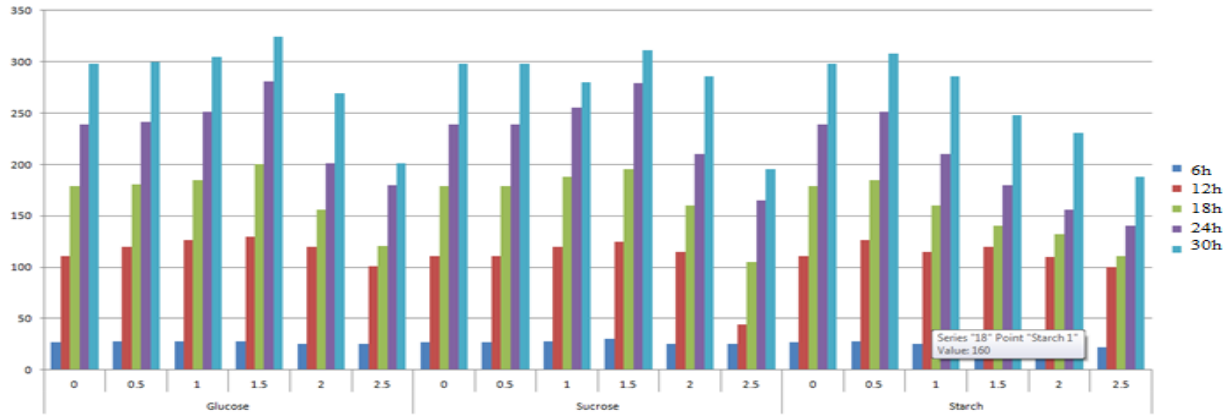

Figure 8: Influence of carbon source on protease production by *B. sonorensis* ASKL-09 showing maximum activity (321 IU/ml) at 1.5 % glucose concentration.

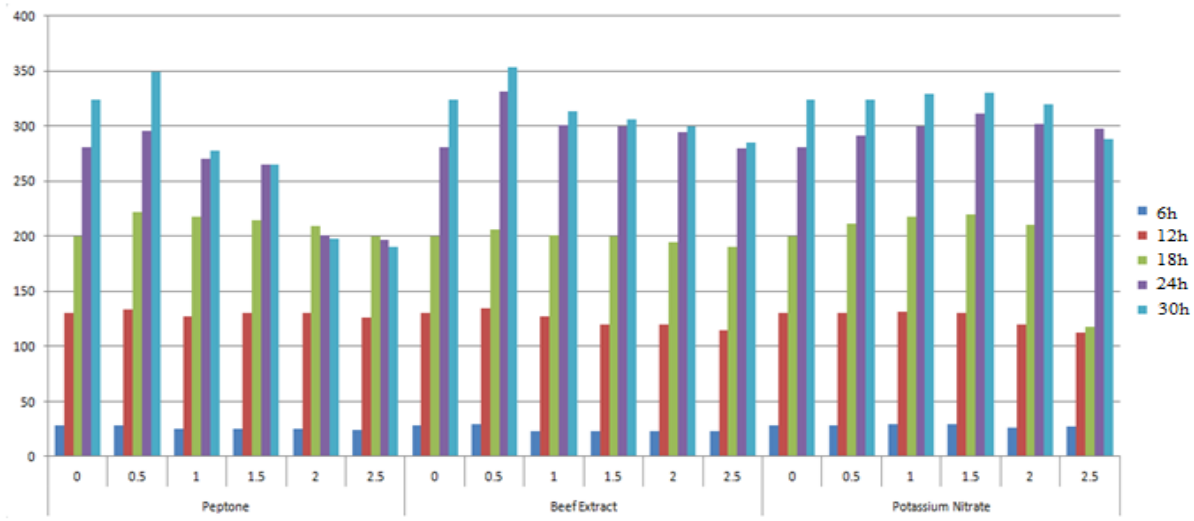

Figure 9: Influence of nitrogen source on protease production by *B. sonorensis* ASKL-09 showing maximum activity (354 IU/ml) at 0.5% beef extract.

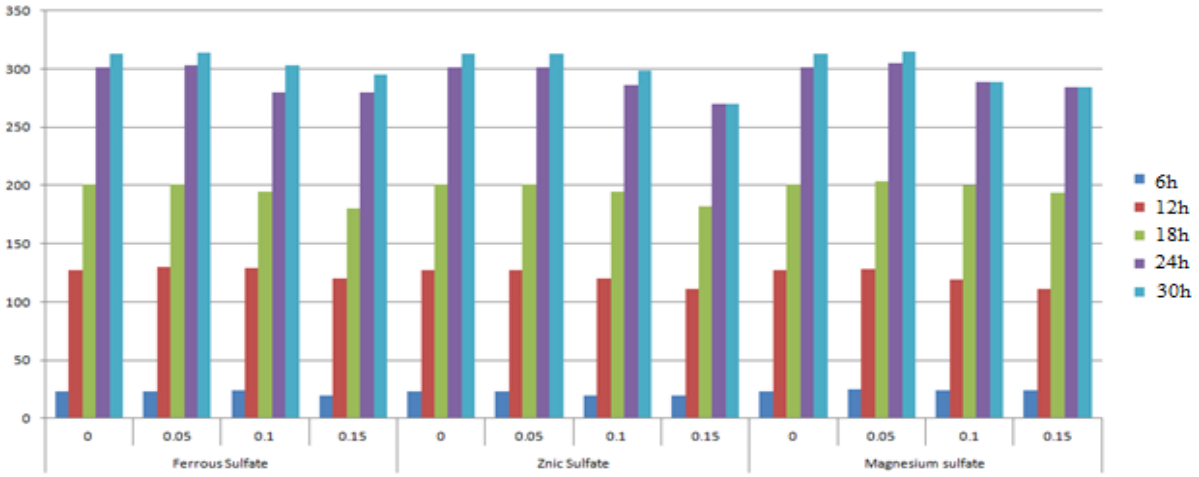

Figure 10: Influence of metal ions on protease production by *B. sonorensis* ASKL-09 showing maximum activity (315 IU/ ml) in magnesium sulphate at 0.05%.

CONCLUSION

A potential protease producing bacteria *B. sonorensis* ASKL-09 was isolated from the soil sample. The bacteria produce thermo alkaline protease. The addition sources such as glucose and beef extract increased the production, while higher concentration of metal ions inhibited the protease production.

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