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Study of Environmental Conditions on Protease Production by *Bacillus* sp. from Lignite Mine of Gujarat

KEYWORDS: *Bacillus*, Enzyme, Lignite, Panandhro, Protease, Folin Phenol

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ABSTRACT *The major cause of acid mine dranage (AMD) or acid rock drainage (ARD) is mining activities of lignite and metal sulphidic mines. The harsh environmental conditions in terms of pH, iron, metals and dissolved solids are responsible for damage of surrounding aquatic and terrestrial ecosystem. Lignite mine ARD of Panandhro showed pH 2.0. Proteases are class of enzymes, which occupy key position in various commercial fields. Protease producing gram positive bacterial isolate belonging to genus Bacillus was isolated from this extreme ecosystem and was used for protease production study under environmental parameters such as aeration and agitation, medium pH and incubation temperature. The isolate showed optimum growth in well aerated condition. The optimum pH and temperature were 7.2 and 37°C, respectively.*

I. Introduction

Proteases constitute one of the most important groups of industrial enzymes. It accounts for nearly 60% of the total market (Rao et al., 1998; Nascimento and Martins, 2004; Kuberan et al., 2010), which leads to extensive research for proteases from various sources for many years. Proteases are also known as proteinases or proteolytic enzyme. Proteases are enzymes that hydrolyze proteins into short peptides or free amino acids. It belongs to class hydrolases, which catalyze the reaction of hydrolysis of various bonds with the precipitation of a water molecule (Palsaniya et al., 2012). Microbial proteases are more significant compared with animal and plant proteases (Kumar et al., 2012). Microbial proteases are good source of enzymes due to their broad biochemical diversity, rapid growth, less space required for cultivation and ease of genetic manipulation to obtain new enzymes for various applications (Singhal et al., 2012). In microbial source bacterial proteases are preffered over fungal proteases. Microorganisms most commonly used for production of proteases includes species of genera *Bacillus*, *Aspergillus*, *Mucor* and *Rhizopus*. The genus *Bacillus* produces a large number of extracellular enzymes, many of which are of considerable industrial importance (Rao et al., 1998). Various studies have been reported for optimization for growth conditions for protease "Production"by various microorganisms (Singhal et al., 2012; Palsaniya et al., 2012). Statistical analysis of the results by SPSS 16.0 is also earlier reported (Varjani et al., 2014).

The research work was focused on optimization of environmental parameters for protease production by indigenous *Bacillus* sp. isolated from waste water sample of Panandhro Lignite Mine of Gujarat. Protease assay was performed by Folin Phenol method. Statistical analysis of the results was carried out by SPSS 16.0. All analysis were performed in triplicates and results are represented as mean ± standard deviation (s.d.).

II. Materials and methods

2.1. Sample collection

Waste water sample (2.0 liter) was collected in sterilized plastic carbouys with technical assistance of mining authorities of Panandhro Lignite Mine situated in Kutch, Gujarat.

2.2. Isolation and purification of microorganism

Collected waste water sample was streaked on casein agar plate (s) and incubated for 72 hours at room temperature. After every 24 hours incubation results were noted as ratio of colony diameter to zone of casein clearance, the colony giving the highest ratio in shortest time was selected as potential protease producer and further studies were carried out. The selected isolate was activated and purified by subsequent transfer on nutrient agar slants.

2.3 Study of environmental conditions on protease production

All experiments were carried out for 72 hours, every 24 hours sample was removed from flasks and enzyme assay was performed to check protease activity against respective blank sample. For each experiment blank/control flask was kept without inoculum.

2.3.1 Effect of aeration and agitation

To check the better condition for growth of an organism, it was allowed to grow in static condition as well as in a shaking condition (150 rpm). The set was kept in 250 ml flasks with a working volume of 100 ml nutrient broth. It was inoculated with 1% v/v active culture having 10^8cells/ml. One flask was kept on a shaker and another was kept at static condition for 72 hours.

2.3.2 Effect of pH

The organism was allowed to grow in nutrient broth containing varying pH such as 3.0, 5.0, 7.2 and 9.0 to observe influence of pH on its growth. In 250 ml flask 100 ml nutrient broth with various pH viz. 3.0, 5.0, 7.0 and 9.0 was inoculated with 1% v/v actively growing culture containing 10^8 cells/ml. The respective pH was adjusted with 0.2N HCl or 0.2N NaOH. The flasks were incubated at 37°C, 150 rpm for 72 hours.

2.3.3 Effect of temperature

In 250 ml flask 100 ml nutrient broth (pH 7.2) was inoculated with 1% v/v actively growing culture containing 10^8 cells/ml and incubated at different temperatures (°C) (30, 37, 45, 50, 60 and 70) for 72 hours at 150 rpm

2.4 Enzymatic assay for protease

Test sample and respective blank sample were collected

Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

from each flask at 24 hours interval and protease assay was performed by standard Folin Phenol method (Lowry et al., 1951). Modified Palsaniya et al. (2012) method was used for these purpose. One unit of protease activity is defined as the amount of protease which liberates 1µg of tyrosine under experimental conditions. Collected broth from each flask was centrifuged at 5,000 g for 10 min. Supernatant was used as a crude protease enzyme.

A) Reagent Blank: 2 ml substrate (1% Casein) was added to 1 ml respective Nutrient broth blank, and incubated for 10 min. at room temperature. Then 8 ml 5 % TCA (Tricloroacetic acid) was added to this incubated mixture and was incubated again at room temperature for 30 min. The reaction mixture was centrifuged at 5,000 g for 10 min. at room temperature. The supernatant (1 ml) was used for assay by Folin Phenol method using tyrosine as standard protein. Optical density was measured spectrophotometrically at 750 nm.

B) Experimental: 2 ml substrate (1% Casein) was added to 1 ml of enzyme solution (crude broth i.e. supernatant from fermentation broth) and incubated for 10min. at room temperature. Then 8 ml 5 % TCA (Tricloroacetic acid) was added to this incubated mixture and was incubated again at room temperature for 30 min. The reaction mixture was centrifuged at 5,000 g for 10 min. at room temperature. The supernatant (1 ml) was used for assay by Folin Phenol method using tyrosine as standard protein. Optical density was measured spectrophotometrically at 750 nm.

2.5 Statistical analysis

All experiments used in these study were carried out in triplicates and results are represented as mean ± standard deviation (s.d.). Statistical evaluation for various environmental parameters for protease production was performed by SPSS 16.0.

III. RESULTS AND DISCUSSIONS

Studies have been reported for isolation, screening, optimization and characterization for protease production by various microorganisms from different industrial wastes (Rao et al., 1998; Kuberan et al., 2010; Kumar et al., 2012, Siala et al., 2012; Habib et al., 2012). Protease can be acidic, neutral or alkaline depending on their activities at different pH (Radha et al., 2011). In detergent industry alkalophilic proteases are used, however acidophilic proteases have significant role in leather tanning process, food industry and x-ray films (for removal of sliver) (Habib et al., 2012; Siala et al., 2012). Proteases production is influenced by different environmental parameters such as (incubation temperature and period; quantity of inoculums; medium pH) and nutritional parameters (NaCl concentration; type and concentration of Carbon and Nitrogen source in growth medium) (Singhal et al., 2012).

Among various isolates obtained *Bacillus* sp. giving highest ratio of colony diameter to zone of clearance of casein (3.5 ± 0.06) on casein agar plate at 24 hours of incubation at room temperature was selected as best protease producer and used for futher studies. The influence of environmental parameters viz. static as well as shaking, temperature and pH on protease production by native *Bacillus* sp. were performed in this study. For all graphs in this paper data represents mean ± s.d., n=3; error bars indicate s.d.

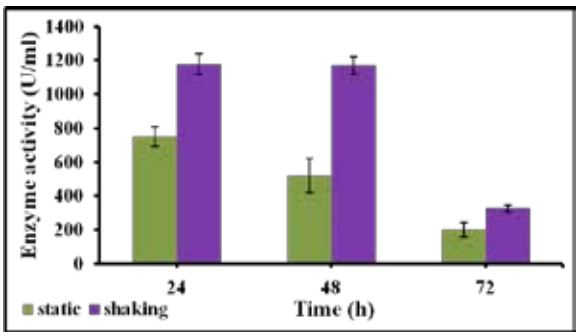

Fig. 1: Influence of aeration and agitation on protease activity

Shaking at 150 rpm for 24 hours was optimal for protesae production however almost same protease activity was observed for incubation at 150 rpm for 24 hours (1180 ± 0.6 U/ml) as well as 48 hours (1170 ± 2.3 U/ml) (Fig. 1). Highest protease activity (1200 ± 0.7 U/ml) was observed at 24 hours for pH 7.2, which decreased as time passes (Fig. 2). Maximum protease activity (1350 ± 1.4 U/ml) by *Bacillus* sp. was observed when it was incubated at 37°C for 24 hours. However these was decreased at increasing time as well as increasing and deceasing temperature (Fig. 3).

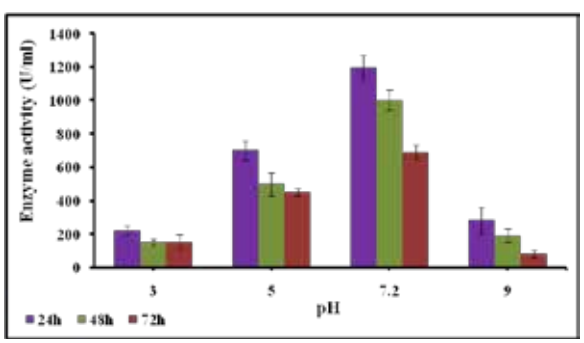

Fig. 2: Influence of pH on protease activity

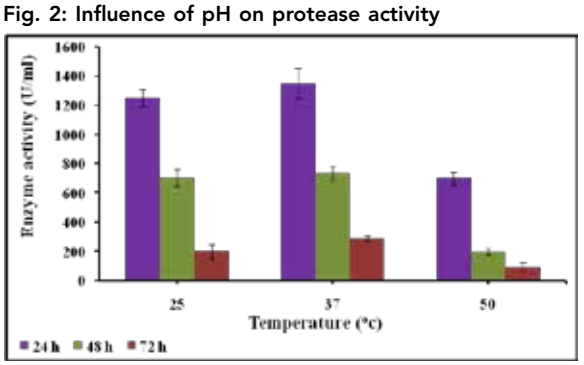

Fig. 3: Influence of temperature (s) on protease activity

Optimization studies revealed that shaking condition (150 rpm), temperature 37°C; pH 7.2; gives best protease activity (1350 ± 1.4 U/ml) at 24 hours incubation, indicating that *Bacillus* sp. used in this study is mesophilic, aerobic protease producer.

IV. CONCLUSION

These studies revealed that indigenous *Bacillus* sp. used in present study is mesophilic and aerobic protease producer. The organism grew better in shaking condition (150 rpm)

Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

at 24 hours incubation giving enzyme activity 1180 ± 0.6 U/ml. The optimum pH for the growth of the organism was 7.2 and least favourable pH was 9.0. The optimum temperature for the growth was 37°C giving 1350 ± 1.4 U/ml protease activity at 24 hours. Further studies regarding optimization of medium components for protease procuction will be envisaged. These organism as well as the product may be of great significance in various industries such as detergent, leather, food, baking and brewing etc.

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