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Nutritional Parameters for Growth Profile Study of Protease Producing Halotolerant Bacteria from Mining Site of Kutch, Gujarat

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Abstract: ***Extensive research on proteases from various sources is thrust area of research as they form a very important part of industrial enzymes. Bacillus sp. producing high yield of protease was isolated from waste water (pH 2.0) of Panandhro Lignite Mine of Kutch, Gujarat. This organism was studied for nutritional parameters viz. as NaCl concentration, substrates and carbon sources for its protease activity. Nutrient broth was used as fermentation medium. Protease activity was measured by Folin Phenol method. Maximum protease activity (1370 ± 0.7 U/ml) was obtained using nutrient broth (pH 7.2) supplemented with 1% w/v Lactose (carbon source), 2% w/v peptone (substrate) when incubated at 37 °C for 24 hours. In addition these protease is halo-tolerant (tolerates 4 % w/v NaCl concentration) which proves its utility in saline areas.***

Keywords: *Bacillus*, Casein**,** Halotolerant, Kutch, Protease, Lactose

1. Introduction

In current scenario enzymes are of great importance and they have dragged world's attention due to their wide range of applications in various fields. The devasting environmental conditions in terms of metals, dissolved solids, pH and iron, are created by mining activities which leads to acid mine drainage (AMD) or acid rock drainage (ARD) (Varjani, 2014). Proteases (EC.3.4) are a group of hydrolytic enzymes which catalyze cleavage of peptide bonds in proteinous substrates. Depending on their mode of action and catalytic mechanism, proteases are divided into four major groups: serine protease (EC.3.4.21), cysteine (thiol) protease (EC.3.4.22), aspartic proteases (EC.3.4.23) and metallo-protease (EC.3.4.24) (Rao et al., 1998). Proteases possess wide commercial applications such as detergents, chemicals, food, baking, brewing, pharmaceutical, silk and leather tanning industries. Other promising applications have been associated in potential biotechnological processes (agricultural and medical) and waste water treatment (Gupta et al., 2002; Sanchez-Porro et al., 2003; Mohapatra et al., 2003). Proteases from microbial sources are preferred over its plant or animal sources since they possess desired characteristics for their biotechnological applications when obtained from microbes. Microorganisms represent an attractive source of proteases as they can be cultured in short time in large quantities and produce high yield of product. In general, microbial proteases are extracellular so that downstream process becomes easier and cost effective (Gupta et al., 1998; Rao et al., 1998; Varjani 2014). The proteolytic activity and growth condition of protease producers are affected by different chemical (media composition) and physical (pH and temperature) parameters as well as by the type of microorganism. However to get a good proteolytic activity screening and identification of potential isolates along with optimization of physical and chemical conditions (temperature, pH and the type & composition of media) for potential protease producing microorganisms is very important (Bizuye et al., 2014)

Presently available proteases are not sufficient to meet industrial demands. So research is going on to search for novel proteases from diverse ecosystems. Microbes from varied habitats have been investigated by many researchers for this purpose. The main objective of this study was to optimize nutritional parameters for protease production by native *Bacillus* sp. isolated from waste water sample of Panandhro Lignite Mine, Gujarat. Protease assay was performed by Folin Phenol method and activity was calculated as U/ml.

2. Materials and Methods

2.1 Collection of sample and selection of protease producer

Panandhro Lignite Mine of Kutch region in Gujarat state was selected as a site to collect waste water sample. Sample collection is described in earlier study (Varjani, 2014). Collected waste water sample was streaked on casein agar plate (s) and incubated for 72 hours at room temperature. Depending upon casein utilization organism was selected for further studies. The detailed procedure is described elsewhere (Varjani, 2014). *Bacillus* sp. was selected as the best protease producer from obtained isolates.

2.2 Preservation of culture and inoculum preparation

Selected *Bacillus* sp. was preserved in 20% v/v sterile glycerol solution at -70 ºC. For routine experiments it was maintained on Nutrient agar slants at 4 ºC and sub-cultured at an interval of 30 days.

For inoculum preparation once again sub-culturing on Nutrient agar slant was performed which was incubated at 37 °C for 24 hours. This was transferred to 100 ml erlenmeyer flask containing 50 ml nutrient broth and incubated for 24 hours at 37 °C at 150 rpm. This overnight grown active

bacterial culture contains 10^8 cells/ml, 1.0 % v/v of this activated culture was used as inoculum.

2.3 Fermentation medium

100 ml nutrient broth in 250 ml erlenmeyer flask was used as fermentation medium. Components of nutrient broth (g/1): peptone 10.00, beef extract 3.00, NaCl 5.0, pH 7.2. The medium was sterilized in autoclave at 121 °C for 15 min. and cooled. All test flasks were inoculated with 1.0% v/v inoculums, whereas blank/control flasks were kept without inoculum for each experiment. The flasks were incubated at 37 °C, 150 rpm for 72 hours. At every 24 hours interval sample was withdrawn from flasks and enzyme assay was performed to check protease activity against respective broth blank.

2.4 Nutritional parameters optimization for protease production

Fermentation medium was supplemented with various nutrient supplements for different nutritional parameters to be studied.

2.4.1 Effect of NaCl concentration

Nutrient broth already contains 0.5% w/v NaCl, additional NaCl (% w/v) 0.0, 0.5, 1.5, 2.5, 3.5 and 4.5 was added in fermentation medium so that final concentration of NaCl (% w/v) become 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 in it.

2.4.2 Effect of substrate

To study metabolic capabilities of organism various substrates like casein (1% w/v), casein (2% w/v), milk (2% v/v) and peptone (2% w/v) were used to study their effect on protease production by replacing 1% w/v peptone in fermentation medium. Milk was sterilized separately because of denaturation of protein medium/broth colour changes.

2.4.3 Effect of carbon source

Effect of various carbon sources such as glucose, manitol, lactose and sucrose at 1% w/v concentration was investigated in fermentation medium. Additional 1% w/v peptone was added to achieve 2% w/v peptone in nutrient broth.

2.5 Protease enzyme assay

The withdrawn culture medium (test sample and respective blank sample were collected from each flask at 24 hours interval) was centrifuged at 5000 rpm for 15 min to obtain the crude extract, which served as crude enzyme source. Protease assay was performed by standard Folin Phenol method (Lowry et al., 1951). Modified Palsaniya et al. (2012), method was used for this purpose.

2 ml substrate (1% Casein) was added to 1 ml respective Nutrient broth blank/test sample i.e. crude enzyme, and incubated for 10 min at room temperature. After 10 min, reaction was terminated by addition 8 ml 5 % TCA (Tricloroacetic acid) and this mixture was incubated at room temperature for 30 minutes. 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. Briefly the process followed for this purpose is: 1 ml of supernatant was added to 5 ml alkaline reagent. This was preceded by addition of 0.5 ml Folin Ciocalteau Reagent. After 30 min, developed colour was measured at 750 nm against tyrosine as standard protein (Assay tube tyrosine was prepared in same manner). One unit of protease activity is defined as the amount of protease which liberates 1µg of tyrosine under experimental conditions.

2.6 Statistical analysis

Determination assay for each protease activity parameter were carried in triplicates [results are represented as mean or mean ± standard deviation (s.d.)] to minimize percentage of error and to achieve significant data. SPSS 16.0 (for windows) and Microsoft Excel 2007 were used for the statistical evaluation and graphical representations of results.

3. Results and Discussions

Wide range of fungi (genus *Aspergillus, Mucor* and *Rhizopus*) and bacteria (genus *Clostridium, Bacillus, Salinivibrio, Halobacterium, Natrialba, Streptomyces* and *Pseudomonas*) are reported to produce proteases (Kuberan et al., 2010, Singhal et al., 2010; Bizuye et al., 2014). Among bacteria *Bacillus* sp. (B*acillus licheniformis, B. firmus, B. subtilis, B. alcalo, B. stearothermiphilus* and *B. thuringiensis*) produces large proportion of commercially available proteases (Kuberan et al., 2010).

Protease producing *Bacillus* sp. was isolated from extreme aquatic ecosystem (pH 2.0) of Panandhro Lignite Mine, Kutch, Gujarat is investigated for protease production under various nutritional parameters for viz. NaCl concentration, substrates, and carbon sources. *Bacillus* sp. used in this study showed optimum protease activity (1260 ± 1.0 U/ml) in 24 hours when grown on 0.5% w/v NaCl concentration, however considerable activity (900 ± 0.4 U/ml) in 24 hours was noted at 4% w/v NaCl (Fig. 1). For all graphs in this study results are mean of three replicates and error bars indicate standard deviation (s.d.).

Majority of industrial processes are accomplished under harsh conditions such as pH, temperature and salinity, it would be of great importance to have microbial sp. as well as their product that demonstrate optimum activity or tolerance at wide ranges of pH, temperature and salt concentration (Ventosa et al., 1998). *Bacillus* sp. used in this study is showing good protease activity at 4% w/v NaCl concentration, indicate its Halotolerant nature.

The effect of various substrates on protease activity is represented fig. 2. The organism was able to grow in various substrates like 1% casein**,** 2% casein, 2% Milk**,** 2% Peptone. The highest protease activity was obtained at 24hours in 2% Peptone water (Fig. 2).

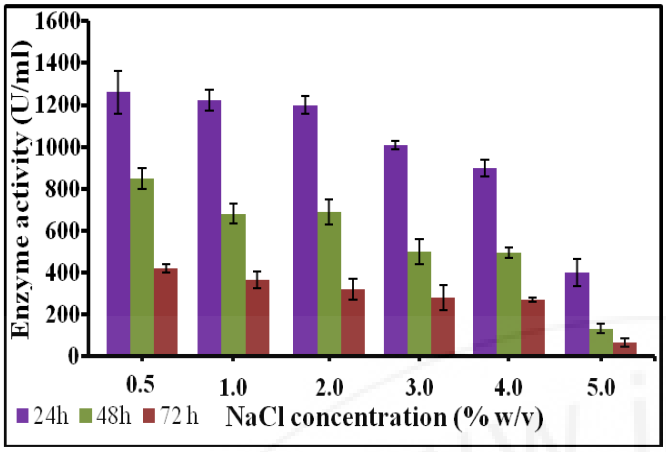

Figure 1: Effect of increased NaCl concentration on protease activity

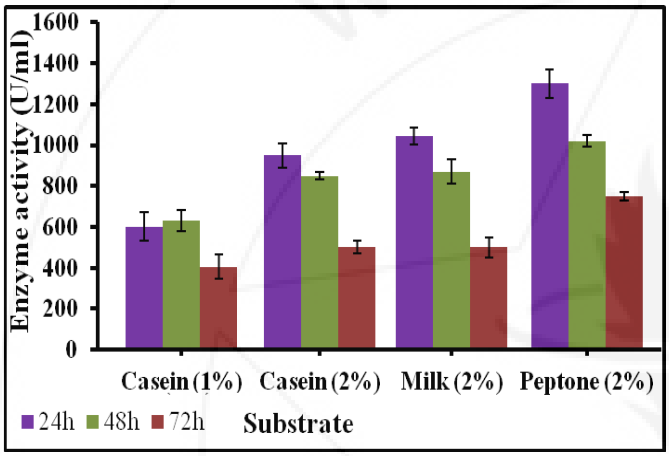

Figure 2: Effect of substrate on protease activity

Lactose was found to be the most preferred carbon source among the tested sugars under the experimental condition for protease activity. 1370 ± 0.7 U/ml protease activity was noted for 24 hours incubation (Fig. 3). For almost all experiments protease activity was decreased with time increase.

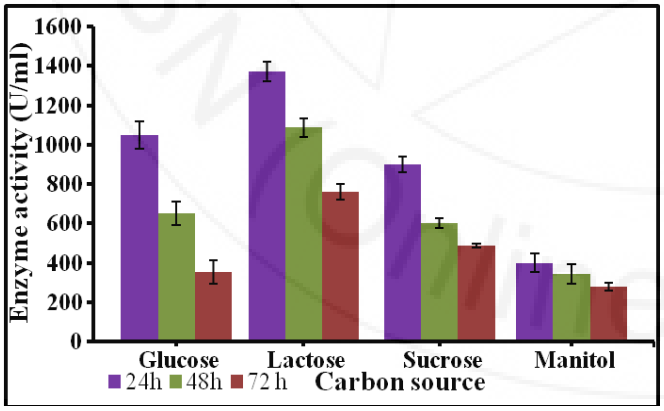

Figure 3: Effect of carbon source on protease activity

Optimization of fermentation medium for maximum protease production was performed by Palsaniya et al. (2012). They have isolated alkaline protease producing *B. subtilis* from soil samples with optimum protease production at 37°C, pH 10, Glucose as carbon source and Peptone as nitrogen source. They also reported Soycake and Calcium chloride as stimulators of protease production. Kuberan et al. (2010), have isolated two *Bacillus* sp. named tk1 and tk2 from salt pan in Tuticorin, Tamilnadu, India. Maximum growth and protease production was reported at 5% (w/v) NaCl however marginal growth without enzyme production was observed evident in absence of salt. Similar results for NaCl concentration are reported for *Bacillus* sp. in present study. Highest growth and protease activity was noted at 0.5% (w/v) lactose followed by fructose for *Bacillus* tkl and glucose followed by fructose for *Bacillus* tk2. Highest activity at pH 8.0 and 35 °C for 48 hours incubation was noted. They also reported that inoculums volume plays vital role in protease production and growth of the bacterial culture. From the results of Kuberan et al. (2010), study and the results of our study it can be said that *Bacillus* tk1 showed more similar results than that for *Bacillus* tk2 for carbon source utilization.

4. Conclusion

From the present study it can be concluded that optimized conditions for protease activity of the *Bacillus* sp. are: fermentation medium Nutrient broth, pH 7.2, temperature 37 °C, salinity 0.5% w/v, incubation time 24 hours, inoculum volume 1% v/v, shaking 150 rpm, carbon source 1% w/v Lactose, substrate 2% w/v peptone. When organism is grown under such experimental conditions it showed optimum protease activity (1350 ± 1.4 U/ml). Also this organism is halotolerant in nature tolerating 4% w/v NaCl concentration. More applications of this protease need to be explored. Detailed characterization and applications of this enzyme in various industries will be envisaged along with molecular characterization of the isolate itself.

5. Acknowledgement

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