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PRODUCTION AND OPTIMIZATION OF PROTEASE FROM *BACILLUS LICHENIFORMIS* NCIM 2044

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ABSTRACT

Proteases are proteolytic enzymes which is an essential constituent for all forms of life on earth. Bacteria are most important protease producers with the genus Bacillus being the most prominent source because of their ability to produce large amount of protease having significant proteolytic activity and stability at high pH and temperature. Culture conditions play significant role on growth and production of protease by bacteria. The present work deals with the optimization of culture conditions required for protease production by Bacillus licheniformis (NCIM No.2044) and to compare two nitrogen sources for the maximum production of protease. Maltose was selected as the best carbon source, as it showed a significant maximum production of protease, also peptone and meat were selected as optimum nitrogen source .pH 12 was found to be optimum pH for the production of protease. The optimum incubation time for the maximum production of protease enzyme from Bacillus licheniformis is 72 hrs. Maximum enzyme activity was observed in the presence of Ba^{2+}.Among the two nitrogen sources selected maltose with peotone showed the maximum activity in comparision with maltose with meat extract.

Key words*: Bacillus licheniformis, maltose, meat extract, protease, peptone.*

INTRODUCTION:

Proteases are the most important group of enzyme produced commercially [1]. Protease enzyme accounts for approximately 40% of the total industrial enzyme market [2]. Proteases or proteolytic enzymes catalyze the cleavage of peptide bonds in proteins [3].They conduct highly selective and selective modification of proteins i.e.zymogenic form of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, processing and transport of secretory protein across the membrane. They catalyze important proteolytic steps in tumor invasion or in infection cycle of a number of pathogenic microorganisms. Their involvement in the life cycle of disease causing organisms has led to become a potential target for developing therapeutic agents against fatal disease such as cancer and AIDS [4]. They have extensive application in a range of industrial products and processes including detergents, food, pharmaceuticals, leather, silk and for silver extraction from used x-rays films [5,6].

Proteolytic enzymes are found in all living organism and they are important for the cell growth and differentiation. They are found in several microorganisms such as protozoa, bacteria, yeast and fungi. The inability of the plant and animal proteases to meet the current world demands (due to extensive use in food, pharmaceutical and detergent industry) has led to an increase interest in microbial protease [7].Among the various microbial sources available for the production of protease enzyme bacteria are the most prominent source [8]. *Bacillus* species are found to dominate the industrial sector [9]. Bacillus licheniformis strains are listed in the third edition of Food Chemicals Codex(1981)

as sources of carbohydrase and protease enzyme preperations used in food processing[10].The aim of the present investigation is to select medium components for the optimal production of protease from *Bacillus licheniformis*.

MATERIALS AND METHODS: *Bacillus licheniformis* with NCIM 2044, was procured from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. The media components were purchased from Hi Media Laboratories, Mumbai, India. The culture was routinely maintained on Nutrient agar slants and subcultures every four weeks.

Protease Production in shake flask fermentation and assay of protease activity: *Bacillus licheniformis* was cultured in a medium containing the following in gradients (g/l): Carbon source1% (w/v),Nitrogen source,0.5%(w/v), Potassium dihydrogen phosphate (0.5g),Sodium carbonate (10g) with pH(8.0).Incubate at 37^{o} for 72 hours at 200 rpm. The culture broth was harvested and centrifuged at 8,000 rpm for 20 minutes. Cell free supernatant was used for measuring the protease activity. Protease enzyme activity was done using casein as substrate. One unit [11] of enzyme will hydrolyze casein to produce color equivalent [12] to 1.0 µmole (181 µg) of tyrosine per minute at pH 8.0 at 37 °C (color by Folin & Ciocalteau's reagent). The µmoles of tyrosine equivalents liberated were calibrated by using the standard curve.

Optimization of carbon, nitrogen and pH: The optimum production of protease was determined using different carbon sources at 1% (w/v), nitrogen sources at 0.5 % (w/v) and with different pH. The carbon sources used for the present include glucose, starch, lactose and maltose. Different nitrogen sources include yeast extract, meat extract, beef extract and peptone. After selecting the best carbon and nitrogen source for the optimum production of protease, different pH was tried ranging from pH 8-12 to select the optimum pH.

Optimization of incubation time: Different incubation periods (24, 48, 72 and 96 hours) were set up was to determine the optimum incubation time required for the production of protease. After respective incubation time, measurement of enzyme activity was done.

Effect of metal ions: To study the effect of various metal ions, on protease activity, enzyme was incubated with 5mM of metal ions (Ca^{2+}, Ba^{2+}, Sr^{2+}, Zn^{2+} and Hg^{2+}) at 40°C for 30 minutes and the enzyme activity was measured .

Statistical Analysis: The data was analysed using student t test and Analysis of Variance (ANOVA) using the statistical software Graph Pad Prism5.

RESULTS AND DISCUSSION:

For the growth of microbial population, carbon is considered to be an important source. However, the utilization of the carbon source differs from species to species .For the present study, 1% of different carbon different sources namely lactose, maltose, starch and glucose were supplemented with the medium. A significant difference was observed among all the treatments. Results from figure 1, indicates that among the four carbon sources used, maltose showed a significant increase in protease activity when compared with other sources. Starch showed the minimum protease activity among the four nitrogen sources. Hence maltose was selected as the optimum carbon source for the production of protease from *Bacillus licheniformis*. *Bacillus* species studies showed that maximum production of protease was achieved with maltose as a carbon [4] which was similar to the present study. Different species of *Bacillus* uses different carbon sources. *Bacillus alcalophilus* uses maltodextrin as a carbon source [13] while *Bacillus subtilis* strain AG-1and EAG-2 uses glucose as a carbon source [14] and *Bacillus coagulans* uses glucose asparagine medium as carbon source[15].

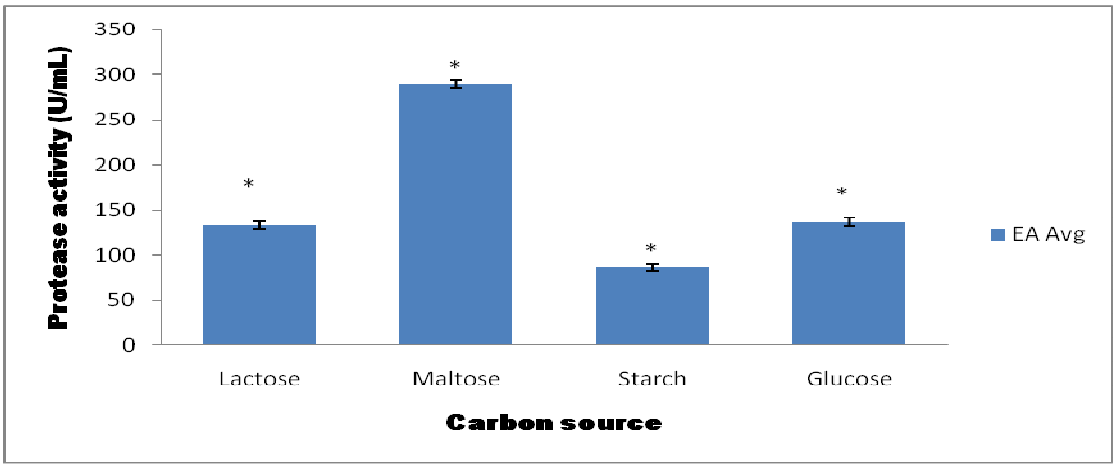

Fig: 1 Effect of different carbon sources on protease production. Results represent the mean of triplicate analysis and bar indicates ± standard deviation. EA Avg. represents average enzyme activity. *significant at $p<0.05$

OPTIMIZATION OF NITROGEN SOURCE:

Nitrogen sources affect the enzyme secretion [16,17]. In bacteria, nitrogen sources are metabolized to produce amino acids, nucleic acids, proteins and cell-wall components. Although requirement of complex nitrogen sources is emphasized for effective protease production in general, efficacy of substrate utilization varies from strain to strain [18]. Among the various nitrogen used for the present study significant maximum protease production was obtained from peptone followed by meat extract as shown in figure 2.While yeast extract showed a significant decrease in protease production .Different carbon sources have different impact on the production of protease enzyme from *Bacillus* species. *Bacillus alcalophilus* uses yeast extract as a nitrogen source [13] .*Bacillus subtilis* strain AG-1and EAG-2 uses casein with yeast extract as a nitrogen source [14]. *Bacillus cereus* strain CA 15, showed its best production of protease with nitrogen source as soluble starch at 1% concentration [19]. Since the highest protease was activity was achieved with peptone followed by meat extract, a comparative study was carried out on both peptone and meat extract to find the highest protease production using maltose as the carbon source.

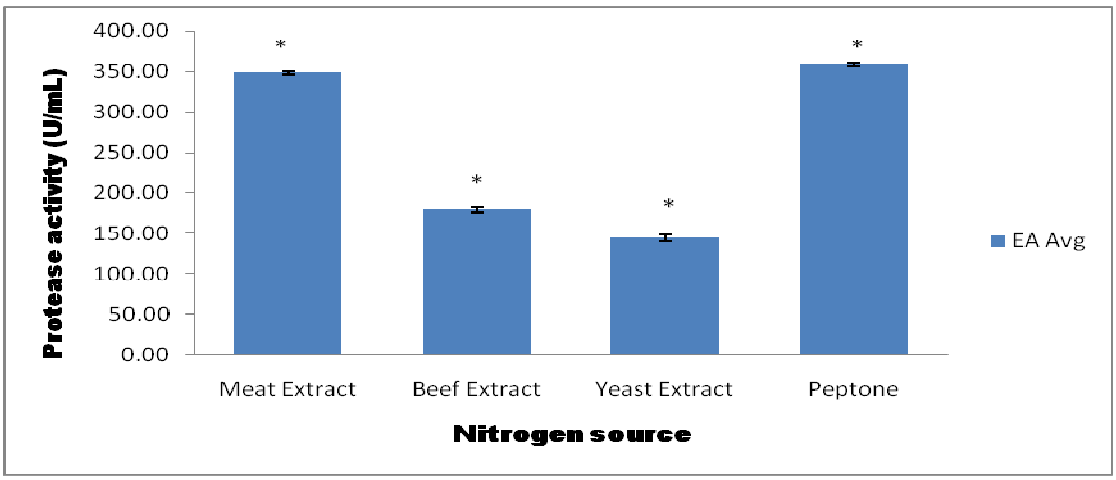

Fig: 2 Effect of different nitrogen sources on protease production.Results represent the mean of triplicate analysis and bar indicates ± standard deviation. EA Avg. represents average enzyme activity.

OPTIMIZATION OF PH: Among the different pH used, pH 12 was found to be optimum pH for the production of protease (Table 3). Hence, it can be said that the protease produced is alkaline in nature and statistically significant. The optimum pH of *Botrytis cinerea* is 6.5 as studied by Ferid Abidi *et al[20]*. While Anissa Haddar *et al*, [21] reported that optimum pH of *Bacillus mojavensis* A21 is 8.0-11.0. Also the optimum pH for the maximum production of protease from *Bacillus licheniformis* NH1 strain was 10.0 on the basis of the work done by Noomen Hmidet *et al* [22]. Usharani and Muthuraj [23] reported that the optimum pH for the protease production from *B. latrosporus* was pH 7.

Table: 3 Effect of pH on protease production.

pH	Protease Activity (U/mL) (Maltose with Peptone)	Protease Activity (U/mL) (Maltose with Meat)
8	*176.66± 3.14	*147.78±4.66
9	*207.78± 3.85	*187.78±5.75
10	203.33± 3.87	*182.22±3.67
11	*181.11±2.81	*180.00±4.70
12	*282.22±3.35	*236.66±4.41

Results represent the mean of triplicate analysis and bar indicates ± standard deviation. *significant at $p<0.05$

OPTIMIZATION OF INCUBATION TIME:

From the table 4, it is clear that the optimum incubation period for the maximum production of protease enzyme from *Bacillus licheniformis* is 72 hrs. With increase in the incubation period, there was an increase in the protease activity. In this study maltose with peptone showed higher enzyme activity than maltose with meat.Therefore, peoptone with maltose was selected as the best carbon source for the optimum production of protease .Praksam and his co-workers reported that the highest activity of protease was observed at 60 hours of incubation time in *Bacillus* species[24].Similar observation was also reported by Johnvesly [25], [26], [27],Yoeman and Edwards [28].

Table: 4 Protease productions at different incubation time

Incubation time	Average Protease Activity (U/mL) (Maltose with peptone)	Average Protease Activity (U/mL) (Maltose with Meat)
24	*23.33 ± 3.77	*11.11±1.92
48	*98.89 ± 4.44	*63.33±4.81
72	*257.77 ± 5.95	*210.00±4.52
96	*165.55± 5.39	*141.11±5.09

Results represent the mean of triplicates and bar indicates ± standard deviation. *significant at $p<0.05$.

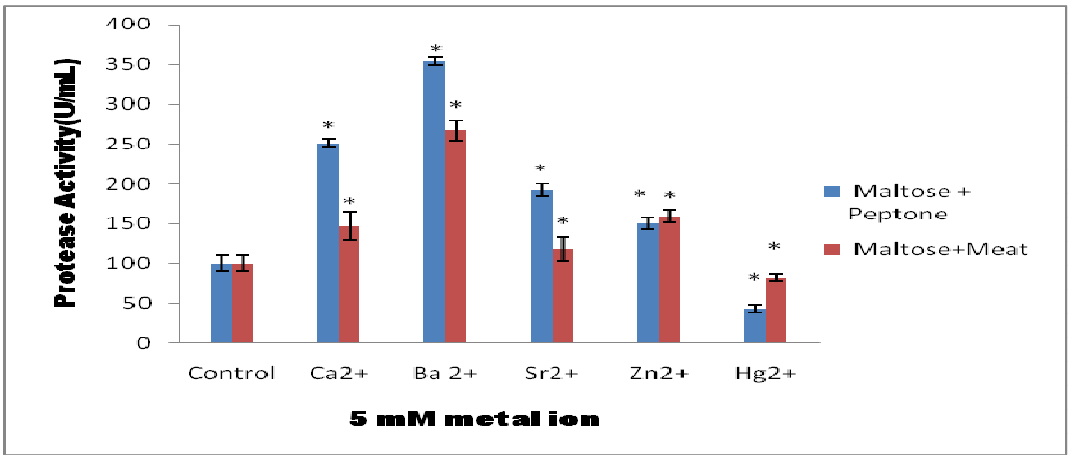

Fig: 5 Effect of metal ions on protease activity**.** Results represent the mean of triplicates analysis and bar indicates ± standard deviation. *significant at $p<0.05$

From the above figure 5 it is clear that, in the presence of barium chloride, the protease enzyme significantly showed maximum activity for maltose with peptone than maltose with meat as compared to other metal ions. Here, control means enzyme without any metal ion and its activity was taken as 100 %. Generally, metal ions are proved to increase the production of protease. Here, metal ions were incubated with the crude enzyme extract and then the enzyme activity was measured. The above tabulated result indicated that except mercuric chloride, other metal ions showed an increase activity in protease production . As compared to control, the presence of metal ions proved to show a significant increase the protease activity. In *Bacillus alcalophilus*, calcium chloride was found to give the maximum protease activity (Kun Cheng,2010). Mabrouk and his coworkers reported that in *Bacillus licheniformis* ATCC 21415 that addition of $CaCl_2$ from 0.01 to 0.07% was optimized for the production of protease[29]. Kun Cheng *et al* [13] reported that for *Bacillus alcalophilus* calcium and magnesium ions increase the protease activity, these cations (Ca^{2+}, Mg^{2+}) have also been reported to increase activity of A21 from *B. mojavensis* A21[30]. While mercury, copper and zinc ions leads to decrease in the protease activity. It is believed that these cations protect the enzyme against thermal denaturation and play a role in maintaining the active conformation of the enzyme at higher temperatures [13]. Fikret Uyar *et al* [19] reported that *Bacillus cereus* strain CA15 shows maximum protease production in the presence of magnesium ion as compared to other metal ions. The optimum concentration of magnesium was found to be 0.6%. It was reported that supplementation of Mg^{2+}, Ca^{2+} and K^{+} salts to the culture medium exhibited slightly better production of protease .

CONCLUSION:

The present study revealed that maltose showed maximum production of protease enzyme hence it was selected as best carbon source. Also peptone and meat was selected as optimum nitrogen source for the production of protease. pH 12 was found to be optimum for the production of protease and optimum incubation time for the maximum production of protease enzyme from *Bacillus licheniformis* is 72 hrs. When compared with other metal ions Ba^{2+} showed maximum enzyme activity .Among the two nitrogen sources selected, maltose with peptone showed the maximum production of protease .

REFERENCE:

[1]Ferrero M.A., Castro G.R, Abate C.M., Baigori M.D., Sineriz F.. Thermostable alkaline protease of *Bacillus licheniformis* MIR 29: isolation, production and characterization,
Appl Microbiol Biotechnol , 1996 ;5:327–32.
[2] Gupta R., Beg Q., Lorenz P.. Bacterial alkaline proteases: molecular approaches and industrial applications, Appl. Microbiol.2002; Biotechnol.59: 15–32.
[3] Godfrey T., West S..Industrial Enzymology, 2nd edition, p. 3, Macmillan Publishers inc, New York,1996.
[4] Rao M.B., Tanksale A.P., Ghatge M.S., Deshpande V.V.. Molecular and biotechnological aspects of microbial proteases, Microbiology and Molecular biology reviews.1998; 62: 597-635.
[5] Kumar C.G., Tiwari M.P., Jany K.D.. Novel alkaline serine proteases from alkalophilic *Bacillus* spp.: purification and some properties, Process Biochem. 1999; 34:441–9.
[6] Cowan D.. Industrial enzyme technology, Trends Biotechnol.1996; 4 :177–178.
[7] Beg Q.K., Gupta R., Lorenz P. Bacterial Alkaline Proteases. Molecular Approaches and Industrial Applications, Applied Microbial. Biotechnol.2002; 59: 15-32.
[8] Sen S., Satyanarayana T..Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40, Indian J Microbiol. 1993; 33: 43-47.
[9]Gupta, R., Beeg Q.K., Loranz P.. Bacterial alkaline proteases: molecular approaches and industrial applications, Appl. Microbiol. Biotechnol.2000; 59(1): 15-32.
[10]Boer, A.S.de, priest F.. Diderichsen, B.,On Industrial use of bacillus licheniformis:a review, Applied Microbiology and Biotechnology.1994;40:595-598.
[11]Anson, M.L.. The estimation of pepsin, trypsin, papain and cathepsin hemoglobin, J.Gen.Physiol. 1938;22,79-89.
[12] Folin O., Ciocalteu V. On tyrosine and tryptophan determination in proteins, J.Biol.Chem.1927;73:627-649.
[13]Kun Cheng,Fu-Ping Lu, Ming Li ,Li-Li Liu ,Xiao-mei Liang..Purification and biochemical characterization of a serine alkaline protease TC4 from a new isolated *Bacillus alcalophilus* TCCC11004 in detergent formulations , African Journal of Microbiology Research,2010; 9(31):4942-4953.

[14] Ghafoor A, Hasnain S.. Production dynamics of *Bacillus subtillis* strain AG-1 and EAG-2, producing moderately alkaline proteases, African J Microbiol. 2009;3(5):258-263.

[15]Ashokan S., Jayanthi C.. Alkaline protease production by *Bacillus licheniformis* and *Bacillus coagulans*, Journal of cell and Tissue Research.2010;10(1):2119-2123.

[16]Mehta V.J., Thumar J.T., Singh S.P.. Production of alkaline protease from alkaliphytic actinomycete, Bioresource Technol.2006;97:1650-4.

[17]Ward, O.P..Proteolytic enzymes In:M.Moo-Young Editor,Comprehensive Biotechnol. 1995;3:789-818.

[18]Gupta A., Khare S.K.. Enhanced production and characterization of a solvent stable protease from solvent tolerant *Pseudomonas aeruginosa* PseA , Enzyme and Microbial Technology.2007;42:11-16.

[19] Uyar F., Porsuk I., Kizil G., Ince Yilmaz E..Optimal conditions for production of extracellular protease from newly isolated *Bacillus cereus* strain CA15, Eur Asian Journal of Biosciences. 5:1-9.

[20] Ferid Abidi A., Ferid Limam., Mazaouki M .Nejib.. Production of alkaline proteases by *Botrytis cinerea* using economic raw materials: Assay as biodetergent. Process Biochemistry, 2008; 43:1202-1208.

[21] Anissa Haddar, Moncef Nasri, Noomen Hmidet. Characterizationn of detergent stable and feather degrading serine proteases from *Bacillus mojavensis* A21, Biochemical Engineering Journal .2009; 47:71-79.

[22] Noomen Hmidet ,El-Hadj Ali Nedra, Haddar Anissa,Kanoun Safia,Sellami Kamoun,Alya Nasri Moncef. Alkaline proteases and thermostable α-amylase co-produced by *Bacillus licheniformis* NH1: Characterization and potential application as detergent additive, Biochemical Engineering Journal .2009; 47:71-79.

[23] Usharani B., Muthuraj M...Production and characterization of protease enzyme from *Bacillus laterosporus*, African Journal of Microbiology Research.2010; 4(11):1057-1063.

[24] Prakasham R.S.,Ch.Subba Rao.,Sarma PN.Green gram husk-an inexpensive substrate for alkaline protease production by *Bacillus* Sp.in solid-state fermentation, Bioresource Technology 2006.97:1449-1454.

[25]Johnvesly B., Manjunath B.R., Naik G.R.. Pigeon pea waste as a novel, inexpensive, substrate for production of a thermo stable alkaline protease from thermo alkalophilic Bacillus sp. JB-99, Bioresour Technol.2002; 82:61-64.

[26] Durham D.R., Steward D.B., Stellwag E.J.. Novel alkaline and heat stable serine protease from alkalophilic *Bacillus* Sp.strain Ex6638, J Bacteriol.1987;169: 2762-2768.

[27] Gessesse, A .The use of nug meal as a low-cost substrate for the production of alkaline protease by the alkaliphilic *Bacillus* sp.AR-009 and some properties of the enzyme, Bioresource Technol.1997; 62:59-61.

[28] Yoemann K.H., Edwards C.. Protease production by *Streptomyces thermovulgaris* grown on rape meal-derived media , J Appl Bacteriol. 1994;77: 264-270.

[29]Mabrouk S.S., Hashem A.M.,Wl-Shayed N.M.A., Ismail A.M.S., Addel-Fattah A.F.. Optimization of alkaline protease productivity by *Bacillus licheniformis* ATCC 21415, Bioresource Technology.1997;69: 155-159

[30] Haddar A., Bougatef A., Agrebi R., Sellami-kamoun, Nasri M.A .Novel surfactant-stable alkaline serine protease from a newly isolated *Bacillus mojavensis* A21,Purification and Characterization. Process Biochem.2009; 44:29-35.