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COMPARATIVE OPTIMIZATION STUDIES OF PROTEASE PRODUCTION BY B. CEREUS NC77, B. SUBTILIS MD2 AND B. AMYLOLIQUEFACIENS MD81

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ABSTRACT Bacteria play a vital role in technology for the production of extracellular enzymes especially proteases which are widely used on an industrial scale in many laundry industries. Soil isolates were screened in casein agar and later shake flask method was used for protease production. Out of 98 isolates, eight were found to be good protease producers. Of which 3 isolates showed maximum protease production viz., *B. cereus* NC77, *B. subtilis* MD2, *B. amyloliquefaciens* MD81 which initially produced proteases of 211U/ml, 175U/ml and 128U/ml respectively. There is increasing demand for enzymes and the need for such economically useful enzymes. An optimization study was carried out using various parameters like incubation period, inoculum size, p^H, temperature, carbon sources, nitrogen sources, metal ions using these three isolates. There was a 4.3 fold rise in protease production with *Bacillus cereus* NC77, an 8 fold rise in production was observed with *Bacillus subtilis* MD2 and a 9 fold rise in production with *Bacillus amyloliquefaciens* MD81 was observed. This protease was used in the removal of blood stains effectively in very less time, low concentrations and hence maybe considered the major concern in many hospital laundries for destaining the blood stained clothes as effective detergents.

KEYWORDS : soil isolates, screening, optimization, production.

INTRODUCTION

The proteases are produced by several microorganisms. These are tremendous source of enzymes due to their broad biochemical variety. Proteins are broken down by microorganisms and they utilize the initiate proteinases produced by microorganisms followed by hydrolysis by peptidases at the extracellular site (Rawling and Barret, 1994). The microorganisms that were found as major industrial protease producers are bacteria of the genera *Clostridium, Bacillus*, *Pseudomonas* (Maal *et al*., 2009). Although various microorganisms were used to produce protease, the enzymes produced by the *Bacillus* genera were so far the most significant group of enzymes formed commercially (Ferroero *et al*., 1996). Most of the proteases produced are bacterial proteases and these are mostly significant to animal and fungal protease (Ward *et al*., 1995). With respect to bacteria, *Bacillus* species are precise producers of extra-cellular protease (Priest *et al*., 1999).

MATERIALS AND METHODS

Sample Collection and Screening

Soil samples were collected from wastes of milk booths, chicken and meat centres from different areas of Hyderabad, Telangana. The samples were screened for protease producing bacteria by taking one gram of soil sample and dispensing in 10ml of sterile distilled water and performing serial dilution. 0.1ml of 10^{-3} dilution was plated on casein agar plates and incubated at 37 ^{0}C for 24h. Out of 98 isolates, eight were found to produce good quantity of protease. *B. cereus* NC77, *B. subtilis* MD2, *B. amyloliquefaciens* MD81 *were* the organisms that was screened for good enzyme production of 211U/ml,175U/ml,128U/ml respectively . Optimisation studies of culture conditions for the three best producing isolates were done.

Media Preparation

Inoculum Preparation

100ml of inoculum broth medium containing 0.2% glucose, 0.05% casein, 0.05% peptone and 0.05% Yeast Extract and salt solution (containing 0.5% KH_2PO_4, 0.2% $MgSO_4.7H_2O$ and 0.01% $FeSO_4.7H_2O$) was taken. The pH was adjusted to 7.0 with 1N NaOH or 1N HCl and sterilised at 121 °C for 15 min. The medium was inoculated with a 24 h old bacterial culture and the flasks were kept on a shaker incubator at 37 °C for 24 h at 150 rpm.

Production medium

The production medium was prepared in a 250 ml Erlenmeyer flask. The composition of production medium consisted of 0.5% glucose, 0.5% peptone and salt solution (containing 0.5% KH_2PO_4, 0.2% $MgSO_4.7H_2O$ and 0.01% $FeSO_4.7H_2O$). The pH was adjusted to 7.0 with 1N NaOH / 1N HCl. This medium was autoclaved at 121 ^{o}C for 15 min. 2% overnight grown inoculum was transferred to 98 ml production medium. The flasks are kept on a shaker incubator at 37 ^{o}C for 72 h at 150 rpm. After incubation, the broth culture was spun in a cooling centrifuge at 10,000 rpm for 10 min. The clear supernatant was collected in separate sterile tubes and was assayed for proteolytic activity and further purified.

Protease Assay

Protease enzyme was assayed by Folin's method (Folin and Ciocalteau, 1929). 1 ml of the clear enzyme supernatant was added with 5.0 ml of substrate solution (0.65% casein in 50 mM Potassium Phosphate buffer, pH 7.5) and incubated at 37°C for 10 minutes. To stop the reaction 5.0 ml of110 mM Trichloroacetic Acid Reagent was added with incubation for 30 min. To the above mixture 5.0ml of 500mM Na_2CO_3 and 1.0 ml of Folin-Ciocalteu's solution (1:1 dilution) were added and incubated for 30 min. The absorbance (O.D) was read at 660 nm against blank. Tyrosine was used to obtain standard curve. One unit (U) of protease was defined as the amount of enzyme that would be required to produce 1 µmol of tyrosine in one minute under the defined assay conditions.

Media Optimization

Optimization of medium composition was done to balance between the various media components, thus minimizing the amount of unutilized components at the end of fermentation. In addition, no defined medium has been established for the optimum production of alkaline protease from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme production.

Effect of Incubation Period

The inoculum size of 2% was inoculated and incubated at varying period's viz., 24h, 48h, 72h , 96h respectively in a shaker incubator and the enzyme activity was recorded.

Effect of Inoculum size

The effect of inoculum size was determined by growing the isolate in fermentation media and inoculating in varied inoculum size viz., 1%, 2%, 5%, 10%, and 15% and the enzyme activity was noted.

Effect of pH

The effect of varying pH of pH5.0 to 9.0 on protease production was carried out. The pH was adjusted using 1N HCl and 1N NaOH and the assay were performed to know the enzyme activity.

Effect of Temperature

The effect of temperature was studied by maintaining the medium at varying temperatures of 5 ° C, 20 °C, 30 ° C, 37 ° C, 40 °C, 50 ° C and the protease assay was performed.

Effect of Different Carbon Sources

Optimization of carbon source was done by adding 1% of different sugars like glucose, fructose, starch, sucrose, maltose, lactose to the production, the protease enzyme quantification was done.

Effect of Nitrogen Sources

Production media was optimized with 1% of various nitrogen sources like ammonium sulphate, ammonium nitrate, casein, tryptone, yeast extract and the enzyme activity was recorded.

Effect of Metal Ions on Protease Production

The effect of metal ions on protease production medium was carried out using 10mM of different metal ions like $MnSO_4.7H_20$, $CaCl_2$, $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$, $FeSO_4.7H_2O$.The protease assay was done and activity of enzyme recorded.

RESULTS AND DISCUSSION

Effect of Incubation Period

Different incubation periods viz., 24, 48, 72 and 96 h were included in the study. The results revealed that optimum incubation period was highest protease production for *Bacillus cereus* NC77 2400 U/ml at 72 h, Bacillus *amyloliquefaciens* MD81 produced 800 U/ml at 72 h, while *Bacillus subtilis* MD2 showed maximum protease production of 770 U/ml at 96 h Fig.1.

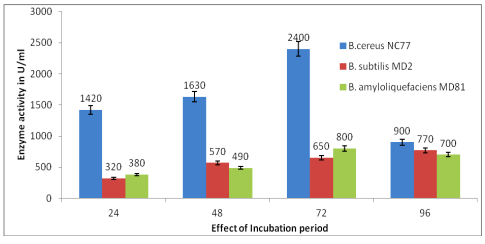

Fig. 1 Effect of Incubation period on *B. cereus* NC77, *B. subtilis* MD2 *B. amyloliquefaciens* MD81

In earlier studies it was seen that *B. subtilis* PE-11 showed maximum protease production at an incubation time of 48 h (Pastor *et al.,* 2001). There were also reports on *B. subtilis* 3411 that gave maximum production at 72h while *Bacillus* sp K-30 showed maximum production at 96h (Naidu *et al.,* 2005; Gibb *et al.,* 1987) which is in relevance with the present study.

Effect of Inoculum size

Inoculum size creates a balance with the available materials that enhance the protease production. The present studies revealed that 5% inoculum size produced maximum protease production of 2650 U/ml in *Bacillus cereus* NC77, while 2% inoculum size produced good protease production of 580 U/ml in *Bacillus subtilis* MD2 and 550 U/ml in *Bacillus amyloliquefaciens* MD81 as shown in Fig. 2.

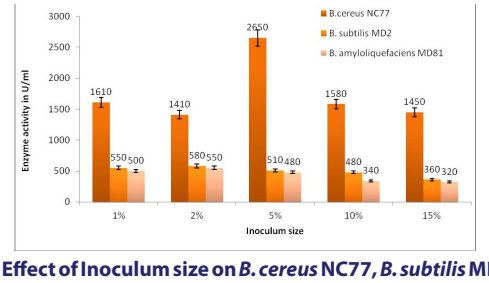

Fig. 2 Effect of Inoculum size on *B. cereus* NC77, *B. subtilis* MD2 *B. amyloliquefaciens* MD81

This result is in accordance with the result reported on 5% inoculum size for optimum protease production by *Bacillus subtilis* (Abusham *et al.,* 2009). The findings are related with the study of Kanekar , 2002 show greatest enzyme activity (167.28 U/ml) of *Bacillus alcalophilus* at 2.0 % inoculum size.

Effect of pH

The physiological character of an organism is dependent on culture. Maximum observed at pH 7.0 in *Bacillus cereus* NC77 which produced 2310 U/ml, *Bacillus subtilis* MD2 produced 650 U/ml and *Bacillus amyloliquefaciens* MD81 produced 540 U/ml respectively as shown in Fig. 3

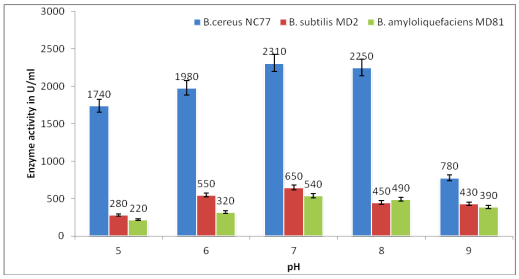

Fig. 3 Effect of pH on *B. cereus* NC77, *B. subtilis* MD2 *B. amyloliquefaciens* MD81

The results are in relevance with previous work of Kanekar *et al.,* 2002; Horikoshi, 1971.

Effect of Temperature

Different incubation temperature viz., 5, 20, 30, 37, 40 and 50 °C were included in the study. The results showed maximum protease production at 37°C in *Bacillus cereus* NC77 which produced 1050 U/ml, *Bacillus subtilis* MD2 produced 650 U/ml and *Bacillus amyloliquefaciens* MD81 produced 600 U/ml respectively as seen in Fig. 4

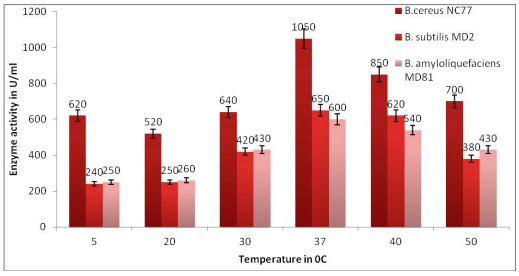

Fig. 4 Effect of temperature on *B. cereus* NC77, *B. subtilis* MD2 *B. amyloliquefaciens* MD81

There were reports indicating that 37 °C temperature for certain *Bacillus* sp (Adinarayana *et al.,* 2002) supports results of study.

Effect of Different Carbon Sources

Effect of carbon sources was tested with 1% of different sugars in the production medium. The results showed maximum protease production with glucose in *Bacillus cereus* NC77 which produced 3160 U/ml, *Bacillus subtilis* MD2 produced 490 U/ml with sucrose and *Bacillus amyloliquefaciens* MD81 produced 1750 U/ml with maltose respectively as shown in Fig. 5.

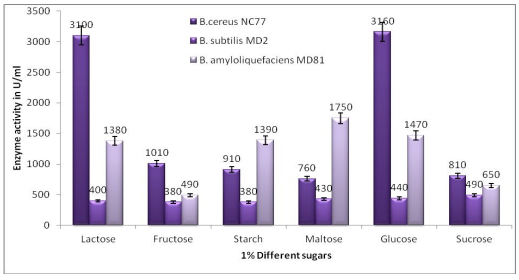

Fig. 5. Effect of carbon sources on *B. cereus* NC77, *B. subtilis* MD2

***B. amyloliquefaciens* MD81**

Lack of glucose in media resulted in dramatic decrease in enzyme production (Gajju *et al.,* 1996; Sonnleitner 1983). Madzak *et al.,* 2000 recorded that the sucrose is good substrate for production of extracellular proteases. The presence of maltose as a carbon source in culture media was also shown to enhance protease production by *Pseudomonas aeruginosa* PseA (Mahanta *et al.,* 2008). The requirement for specific carbon sources differs from strain to strain.

Effect of Nitrogen Sources

Different nitrogen sources like ammonium sulphate, ammonium nitrate, casein, tryptone and yeast extract at 1% concentration was studied. The results showed maximum protease production with tryptone in all the three strains. *Bacillus cereus* NC77 produced 1040 U/ml, *Bacillus subtilis* MD2 produced 1070 U/ml and *Bacillus amyloliquefaciens* MD81 produced 2290 U/ml respectively as seen in Fig. 6.

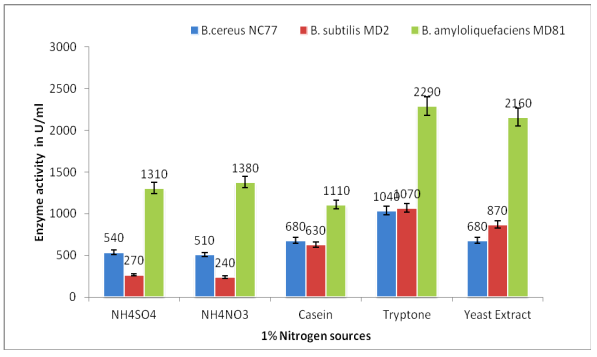

Fig. 6 Effect of nitrogen sources on *B. cereus* NC77, *B. subtilis* MD2 *B. amyloliquefaciens* MD81

It was reported by Phadatare *et al* (1993) that the enhancement of protease production in *Conidiobolus coronatus* by organic nitrogen sources like tryptone, peptone and yeast extract is in relevance with the present study.

Effect of Metal Ions on Protease Production

The effect of 10mM concentration of different metal ions like $MnSO_4.7H_2O$, $CaCl_2$, $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$, $FeSO_4$. $7H_2O$ on protease production was studied. The results showed increase in protease production with Ca^{2+} in *Bacillus cereus* NC77 producing 2830 U/ml and Mn^{2+} enhanced the protease production with *Bacillus subtilis* MD2 and *Bacillus amyloliquefaciens* MD81 producing 670 U/ml and 1780 U/ml respectively as seen in Fig. 7.

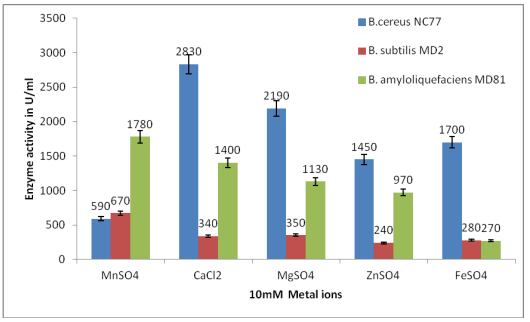

Fig. 7 Effect of metals on *B. cereus* NC77, *B. subtilis* MD2 *B. amyloliquefaciens* MD81

Calcium ion has been reported to enhance protease production in several organisms (Chauhan *et al.,* 2004; Nadeem *et al.,* 2007). Karbalaei-Heidari *et al* (2009) reported that Ca^{2+} Mg^{2+} and Mn^{2+} ions enhanced proteolytic activity.

On optimisation with the above parameters, the protease production in all the three strains viz., *Bacillus cereus* NC77, *B.subtilis* MD2, *B. amyloliquefacience* MD81 produced 902 U/ml, 1360 U/ml, 1140 U/ml respectively as shown in Fig 8.

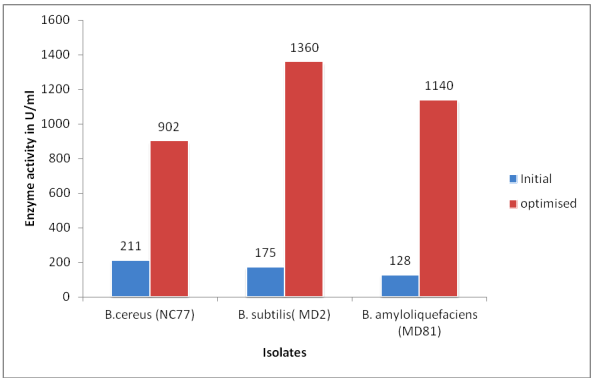

Fig. 8 Enhanced protease production on optimization with *B. cereus* NC77, *B. subtilis* MD2, *B. amyloliquefaciens* MD81 from their initial amounts

CONCLUSION

On optimising with the above parameters, the protease production in all the three strains increased. There was a 4.3 fold rise in protease production with *Bacillus cereus* NC77, an 8 fold rise in production was observed with *Bacillus subtilis* MD2 and a 9 fold rise in production with *Bacillus amyloliquefaciens* MD81 was observed. The characterisation of proteases produced can further be studied. This protease maybe used as detergent in many laundry detergents including hospital laundries.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

REFERENCES

1. Abusham, R.A., R.N. Rhaman, A.B. Salleh, M. Basri. (2009) Optimization of physical factors affecting production of thermostable organic solvent tolerant protease from a newly isolated halotolerant Bacillus subtilis strain R and. Microb. Cell Fact., Vol.8. 10.1186/1475-2859-8-20.
2. Adinarayana, K., Ellaiah, P., (2002); "Response surface optimization of the critical medium components for the production of alkaline protease by a newly isolated Bacillus sp. J Pharm Pharmaceut Sci . 5(3): 272-278.
3. Chauhan, B., Gupta , R. (2004), Application of statistical experimental design for optimization of alkaline protease production from Bacillus sp. RGR-14 Process Biochem. 39 pp.2115–2122.
4. Ferrero, M.A., G.R., Abate, C.M., Baigori, M.D., Sineriz, F. (1996); Thermostable alkaline protease of Bacillus licheniformis MIR 29: isolation, production and characterization. Appl Microbiol Biotechnol., 45: 327-332.
5. Folin. O. and Ciocalteau, V. (1929); Enzymatic Assay of Protease Using Casein as a Substrate, J.Biol. Chem., 73, 627.
6. Gajju, H., Bhalla, T.C., Agarwal, O.H. (1996); Thermostable alkaline protease from thermophilic Bacillus coagulans PB-77, Indian J. Microbiol., 36: 153-155.
7. Gibb, H., Strohl, W.R. (1987);Physiological regulation of protease in Streptomyces peucetius, Can. J. Microbiol. 34: 187-190.
8. Horikoshi, K. (1971); Production of alkaline enzymes by alkalophilic microorganisms and alkaline protease produced by Bacillus No.221. Agric. Biol. Chem. 35: 1407-1414.http://dx.doi.org/10.4014/jmb.1010.10060.
9. Kanekar, P.P., Nilegaonkar, S.S.,.Sarnaik, S.S.,Kelkar. A.S. (2002); Optimization of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. Bioresour, Technol.85: 87-93.
10. Karbalaei-Heidari, H.R., Ziaee, A.A., Schaller, J., Amoozegar, M.A. (2009); Purification and characterization of an extracellular haloalkaline protease produced by the moderately halophilic bacterium, Salinivibrio sp. strain AF-2004. Enzyme Microb. Technol. 40: 266-272.
11. Maal, K.B., Emtiazi, G., and Nahvi, I. (2009); Production of alkaline protease by Bacillus cereus and Bacillus polymixa in new industrial culture mediums and its immobilization, Afr. J. Microbiol. Res., 3 491-497.
12. Madzak, C., Treton, B., and Blanchin-Roland S. (2000); Strong hybrid promoters and integrative expression/secretion vectors for quasiconstitutive expression of heterologous proteins in the yeast Yarrowia lipolytica. J. Mol. Microbiol. Biotechnol. 2: 207-216
13. Mahanta, N., Gupta, A., Khare, S.K. (2008); Production of protease and lipase by solvent tolerant Pseudomonas aeruginosa PseA in solid-state fermentation using Jatropha curcas seed cake as substrate. Bioresour Technol.99: 1729–1735. doi: 10.1016/j.biortech.2007.03.046.
14. Nadeem, M., Qazi, J.I., Baig, S., Syed, Q.U.A. (2007), Studies on commercially important alkaline protease from Bacillus licheniformis N-2 isolated from decaying organic soil.

Turk J Biol, 32, pp. 171–177.
15. Naidu, K.S.B., Devi, K.L. (2005); Optimization of thermostable alkaline production from species of Bacillus using rice bran, Afr J. Biotechnol. 4: 724-726.
16. Pastor, M.D., Lorda, G.S., Balatti, A. (2001); Protease obtention using Bacillus subtilis 3411 and Amaranth seed meal medium of different aeration rate, Braz J. Microbiol. 32: 6-9.
17. Phadatare, S., Deshpande, V., Srinivasan, M. (1993); High activity alkalineprotease from Conidiobolus coronatus (NCL 86-8.20): Enzymes production and compatability with commercial detergents. Enzyme MicrobTechnol. 15, 72-76.
18. Priest. F.G., (1977); Extracellular enzyme synthesis in the genus Bacillus. Bacteriol. Rev., (41), 711-735.
19. Rawling, N.D., Barret, A. (1994); Families of aspartic peptidases, and those of unknown mechanism. Meth. Enzymol. 248: 105-120.
20. Sonnleitner, B. (1983); Biotechnology of thermophilic bacteria growth, products and application in Adv. Biochem. Engi. Biotechnol. edited by A Fiechter (Springer-Verlarg, Berlin) 70-138.
21. Ward, O. P. (1995); Proteolytic enzymes In: M. Moo-Young Editor, Comprehensive Biotechnol. 3:789-818.