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Screening and enhanced production of protease from a thermophilic *Bacillus* species

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Abstract: Thermophilic *Bacillus* species was observed as the best candidate for maximum production of protease. Screening of protease indicated clear hydrolytic zone around culture on casein agar plate. It revealed the ability of culture to release protease extracellularly. Optimization of casein as a carbon source showed maximum production of protease with 1 % concentration. Incubation period of 72 hours was found as optimum for synthesis of maximum concentration of protease that produced 335 U/ml/min. Such high production yield of protease showed its broad potential applications in different biotechnological and industrial processes.

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INTRODUCTION

Proteases are considered as one of the main groups of industrially valuable enzymes with several applications. Proteases can be used in detergent, baking, chess production, de-hairing, waste management, in recovery of silver and as meat tenderizer[1].Several microbial species have been used for the production of different enzymes. Among them, *Bacillus* strains have been investigated for hyper production of alkaline, thermo-stable and broad pH range stable enzymes which mostly have high metabolic diversity[2]. Such strains are also mostly preferred due to short cultivation period, less space requirement and relatively easy genetic modification for production of enzymes with more efficient properties that can be employed for different biotechnological applications[3, 4]. Bacterial proteases are mostly released extracellularly in fermentation medium. Protease from *Bacillus* species have been purified and characterized with high catalytic activity and broad substrate specificity[5, 6].

Proteases contribute more than 65% of total enzyme market and have several biotechnological applications[7]. They are also classified as exo-peptidases and endo-peptidases. Exo-peptidases involve in the catalytic process of proteins and cleave the peptide bonds at N or C terminal of polypeptide chains while endo-type hydrolyze the peptide bonds within polypeptide chain[8].Different fermentation strategies have been investigated for hyper production of protease such as submerged and solid state fermentation. However, submerged fermentation has been used for the maximum production yield of enzymes due to easy nutrients and oxygen availability to the culture as well as less time required[9-11].

In this study, an attempt was made to screen the thermophilic *Bacillus* species for the production of protease extracellularly. Initially, substrate concentration was optimized in fermentation medium and then the effect of temperature was investigated for maximum enzyme production. Enzyme activity was also analyzed on the casein containing agar plate that indicated the proteolytic activity of enzyme for different industrial bioprocesses.

MATERIALS AND METHODS

Different *Bacillus* species were obtained from a culture bank of Department of Biotechnology, University of Karachi, Pakistan. Initially, bacterial isolates were screened for protease production on Luria Bertani (LB) agar plates supplemented with 1% casein. *Bacillus* species which showed high proteolytic activity was selected for further work. Selected *Bacillus* species was preserved on LB agar slants for short term preservation and in 40% glycerol for long term preservation. Culture was maintained by regular sub-culturing on fresh media.

Submerged fermentation strategy was investigated for maximum production yield of protease by *Bacillus* species. Fermentation time and substrate concentration were optimized for maximum production of enzyme.

The influence of different substrate concentrations was examined on the production of protease by inoculating *Bacillus* species in 0-2% casein containing LB broth for 72 hours at 60°C in orbital shaker (120rpm).

The impact of incubation time was analyzed on the maximum production of protease from *Bacillus* species. Culture was inoculated in growth medium and incubated at different temperatures ranging from

24 to 96 hours. After each time interval, fermentation broth was centrifuged at 5,000rpm for 10 minutes at 4°C. Supernatant was used to determine the catalytic activity of enzyme by Kunitz method[12] under standard assay conditions.

The protease activity was determined by Kunitz method[12]. 0.5ml cell free filtrate (CFF) and 0.5ml of 1% (w/v) casein solution (dissolved in 0.1M Tris-HCl buffer having pH-8.0) were incubated at 37°C for 20 minutes.Then reaction was terminated by adding 1.5ml of 0.3M trichloroacetic acid and then centrifugation was performed at 10,000rpm for 10 minutes at 4°C. Optical density was measured at 280nm by spectrophotometer. All enzyme assays were performed in triplicates. One Kunitz unit is defined as "the amount of enzyme which affects a ΔA_{280} of 0.001under standard test condition". For the estimation of protein concentration, a dye binding method[13] was used. Bovine serum albumin (BSA) solution was prepared with 100µg/ml stock concentration. Then, standard curve was prepared by diluting the stock concentration from 10-100µg.the protein concentration was monitored in term of absorbance at 595nm.

RESULTS AND DISCUSSION

Screening of different *Bacillus* isolates indicated the selection of thermophilic *Bacillus* species that confirmed the maximum production of protease by showing clear milky zone around its culture on casein agar plate (Figure 1). Such hydrolytic zone also revealed the extracellular protease production that has potential applications in different industries including leather industry where protease can be used in de-hairing of animal hides[14].

Figure 1: Screening of thermophilic *Bacillus* species on LB agar medium supplemented with 1 % casein.

The effect of substrate (casein) concentration was examined for maximum protease production from *Bacillus* species. It was observed that maximum production was achieved with 1 % substrate concentration and synthesized enzyme with 322 U/ml/min (Figure 2). Concentration above or below optimum decreased the enzyme production. About 287 and 283 U/ml/min of enzyme were observed by incorporating 0.5 % and 1.5 % casein concentration in growth medium. However, 209 U/ml/min was also produced in medium without any substrate that also indicated the constitutive enzyme production from *Bacillus* species. But addition of casein enhanced the enzyme production as compared to media without substrate. Different bacterial species have been reported that require different concentration of casein for the maximum production of proteases. *Bacillus subtilis* showed maximum protease production yield in the presence of 0.03% gelatin as a substrate[15].

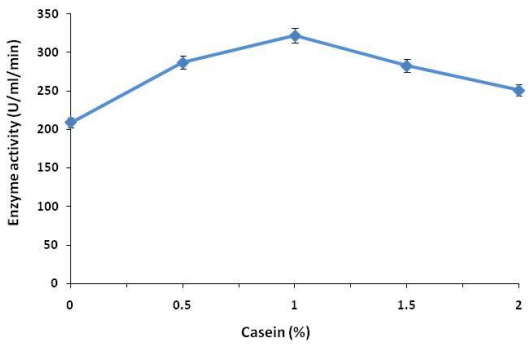

Figure 2: Effect of different concentrations of substrate (casein) on maximum protease production.

The influence of fermentation time was also investigated for the maximum production of protease from *Bacillus* species. It was evident that protease production was increased as time increased and maximum production of enzyme achieved after 72 hours of fermentation period (335 U/ml/min.). After optimum time, about 12 % protease production was decreased when culture was incubated for 96 hours (Figure 3). Different *Bacillus* strains have been reported for different fermentation time of protease. *Bacillus subtilis* synthesized high quantity of protease after 36 hours at 45°C[15]. *Bacillus licheniformis* produced maximum concentration (141.46 U/mg) of protease in the incubation period of 24 hours at pH-8.0 and with 250 rpm agitation[16].

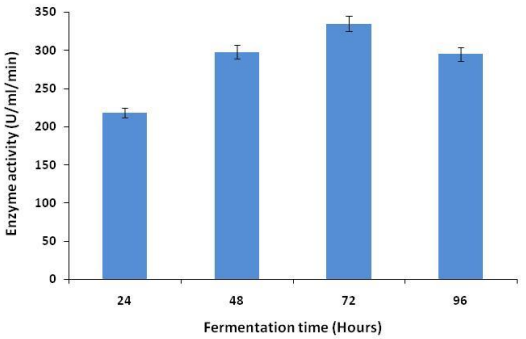

Figure 3: Effect of different fermentation time on maximum protease production.

CONCLUSION

It can be concluded that thermophilic *Bacillus* species was an effective candidate for the maximum production of protease. Initially screening was performed that revealed clear zone of hydrolysis around bacterial isolate. The optimization of substrate concentration and fermentation time indicated 1 % and 72 hours respectively for the maximum production of protease. High production yield of protease suggested its broad potential applications in different industry.

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REFERENCES

1. Gupta R, Beg Q and Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol. Biotechnol*., 2002; 59: 15-32.
2. Bajaj BK and Singh NP. Production of xylanase from an alkalitolerant *Streptomyces* sp.7b under solid-state fermentation, its purification, and characterization. *Appl. Biochem. Biotechnol*., 2010; 162: 1804-1818.
3. Anwar A and Saleemuddin M. Alkaline proteases: A review. *Bioresour. Technol*., 1998; 64: 175-183.
4. Beg QK and Gupta R. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis. Enzyme and Microb. Tech*., 2003; 32: 294-304.
5. Haddar A, Agrebi R, Bougatef A, Hmidet N, Sellami-Kamoun A and Nasri M. Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21: Purification, characterization and potential application as a laundry detergent additive. *Bioresour. Technol.*, 2009; 100: 3366-3373.
6. Maurer KH. Detergent proteases. *Curr. Opin. Biotechnol*., 2004; 15: 330-334.
7. Banik RM and Prakash M. Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*. *Microbiol. Res*., 2004; 159: 135-140.
8. López-Otín C and Bond JS. Proteases: Multifunctional enzymes in life and disease. *J. Biol. Chem.*, 2008; 283: 30433-30437.
9. Hoq MM, Hempel C and Deckwer WD. Cellulase free xylanase by *Thermomyces lanuginosus* RT9; Effects of aeration, agitation and medium components on production. *J. Biotechnol.*, 1994; 37: 49-58.
10. Gomes DJ, Gomes J and Stiener W. Production of highly thermostable xylanase by a wild strain of thermophilic fungus *Thermoascus aurantiacus* and partial characterization of the enzyme. *J. Biotech*., 1994; 37: 11-22.
11. Gouda MK. Purification and partial characterization of cellulose free xylanase produced in solid state and submerged fermentation by *Aspergillus tamarii*. *Adv. Food Sci*., 2000; 22: 31-37.
12. Kunitz M. Crystalline soybean trypsin inhibitors. Part II. General properties. *J. Gen. Physiol*., 1946; 30: 291-301.
13. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*., 1976; 72: 248-254.
14. Vijayaraghavan P, Lazarus S and Vincent SGP. De-hairing protease production by as isolated *Bacillus cereus* strain AT under solid-state fermentation using cow dung: Biosynthesis and properties. *Saudi J. Biol. Sci*., 2014; 21: 27-34.
15. Pant G, Parakash A, Pavani JVP, Bera S, Deviram GVNS, Kumar A, Panchpuri M and Prasuna RG. Production, optimization and partial purification of protease from *Bacillus subtilis*. *J. Taibah. Univ. Sci.*, 2014; http://dx.doi.org/10.1016/j.jtusci.2014.04.010
16. Suganthi C, Mageswari A, Karthikeyan S, Anbalagan M, Sivakumar A and Gothandam KM. Screening and optimization of protease production from a halotolerant *Bacillus licheniformis* isolated from saltern sediments. *J. Genet. Eng. Biotechnol*., 2013; 11: 47-52.