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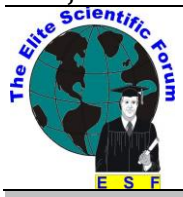

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RESEARCH ARTICLE

Optimal Production of Proteases from *Bacillus subtilis* Using Submerged Fermentation

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ABSTRACT

Proteases are one of the leading groups of enzymes based on their commercial applications. It is demand of the modern era to find an optimized process for maximal production of proteases. In this study, proteases were produced from a bacterial isolate *Bacillus* (*B.*) *subtilis* by using submerged fermentation (SmF), a very economical approach. Growth conditions and supplementary parameters were optimized to enhance protease production. One-way ANOVA, post hoc and LSD tests were applied to analyse the protease production. Optimum conditions for maximum protease production were found to be 48 hours of incubation time; agitation 220 rpm with continuous shaking, pH 7.5; temperature 45 °C; skim milk 2%; yeast sludge 300 µL; ammonium sulphate 0.4%; urea 0.2%; and cane molasses 0.03%. This work succeeded to optimize different physical and nutritional parameters for maximal production of proteases from *B. subtilis*.

INTRODUCTION

Peptidase or proteinase as synonyms of protease (EC 3:4, 11-19, 20-24) belongs to a complex and a very large group of enzymes having commercial importance based on a vast range of applications. They are distinguished in their physical properties including catalytic site and mechanisms, stability, active site, optimum temperature and pH. Among various sources, microbial production of the enzymes is preferred due to their fast growth and simplicity for the generation of new recombinant enzymes with desired properties (Jisha et al., 1992). Bacteria are the most important neutral/alkaline protease producers especially the genus *Bacillus* due to their ability to produce large amount of such enzymes having significant proteolytic activity, and stability even at high pH and temperature (Kumar and Vats, 2010). Among other states of fermentation, liquid state fermentation (also known as submerged fermentation) is very suitable to produce proteases at commercial scale. In SmF, aqueous medium is used in which substrate is suspended or in dissolved form. SmF is preferred for large-scale production of proteases due to better monitoring and ease of handling, and optimizations (Singhania et al., 2010).

The production of the proteases from *Bacillus* (*B.*) *subtilis* by submerged fermentation using skim milk as substrate is reported in this manuscript. Vital parameters like mode of fermentation, pH, temperature, substrate level, and various ionic concentrations were optimized for maximum production of the proteases.

MATERIALS AND METHODS

Chemicals and reagents

In this study, all chemicals and reagents were of analytical grade, purchased from Sigma (USA), Merck (Germany), Scharlau (Spain), Oxide (UK), Thermo Fisher (USA).

Growth of microorganism

Pure culture of *B. subtilis* (MBL-BS-004) was available at Molecular Biochemistry Lab. (MBL), Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan. For inoculation of growth medium, a loopful culture from the slant of *B. subtilis* culture was transferred aseptically into 250 mL Erlenmeyer flasks containing 50 mL of sterile inoculum medium (g/100 mL) Peptone, 1; yeast extract, 0.5; NaCl, 1. The flasks were kept in Sanyo orbital shaker at 37°C and 220 rpm

till the bacterial growth reached up to 10^8 cells/mL in 24 hours.

Production and optimization of protease

Maximum production of proteases was achieved by optimizing different conditions. To get maximum enzyme production, all fermentation conditions/ parameters were optimized including pH (6.5-9.5), temperature (30-50ºC), incubation time (24-72 hours), substrates (skim milk and casein), substrate level (0.5-2.0%), yeast sludge (100-400 µL), ammonium sulphate (0.2-0.5%), urea (0.1-0.25%) and cane molasses (0.3-0.10%), and shaking conditions (stationary, semi-continuous and continuous shaking) by using 4 mL inoculum per 100 mL of growth medium at 220 rpm shaking and 2% of molasses. After centrifugation, the supernatant was subjected to protease assay (Ahmed et al., 2011).

Protease assay

As a substrate, skim milk was used for the measurement of protease activity (Kumar and Vats, 2010). A test tube containing 5 mL of 2% substrate was incubated in dry bath at 37ºC for 5 minutes followed by addition of 1 mL of crude enzyme extract and then incubated further for 30 minutes. The reaction was stopped with the addition of 2 mL of 10% TCA (trichloro acetic acid) followed by vertexing and incubation for 30 min. The solubilized/hydrolysed skim milk was separated through Whatman filter paper and subjected to enzyme assay of spectrophotometer at 275 nm. One-unit of enzyme activity is defined as 1 µmol. release of tyrosine from 1 mL of crude enzyme after one-hour incubation.

Statistical analysis

Each experiment was carried out in triplicate. To check the significance level and reliability of data of the production of proteases it was tested through one-way ANOVA, post Hoc test and LSD test (Sundararajan et al., 2011).

RESULTS AND DISCUSSION

In this study, protease production was enhanced by optimizing different physical and nutritional parameters. Optimization of each parameter was based on the level of enzyme activity.

Incubation period and culture conditions

Incubation period affects growth and protease production (Akcan, 2012). In the present study, a continuous increase in protease production was observed from beginning to 48 h (Fig 1). Three modes of fermentation viz stationary, semi shaking and continuous shaking were tested and flasks were picked after every 24 hours till 72 hours. Maximum proteases were observed after 48 h from continuous shaking at 220 rpm (Fig. 1). The downturn in enzyme activity detected before and after 48 h of incubation might be owed to denaturation and/or disintegration of protease because of interactions with alternate compounds in the fermented medium (Ozcelik et al., 2014; Nassar et al., 2015).

Effect of pH

Change in pH greatly influences the level of enzyme production (Sarkar et al., 1998). Production of protease was observed at varied pH values ranging from 6.5 to 9.5. Highest protease activity (34.43 U/L) was observed at pH 7.5 after 48 h (Fig.2). It shows that the enzyme falls in neutral and alkaline protease family. This is logical with the property of tyrosine family (Wang et al., 2008; Thuy and Bose, 2011). Savitha et al. (2011) reported that ionization of amino acid was affected by pH which further influenced the enzyme activity to primary and secondary structure of enzymes. Dorcas and Pindi (2016) reported optimal activity of bacterial proteases at pH 7.0. Tari et al. (2006) and Liu et al. (2010) also found the pH 8 and 8.25, respectively to be optimal for maximal production of proteases from *Bacillus* species.

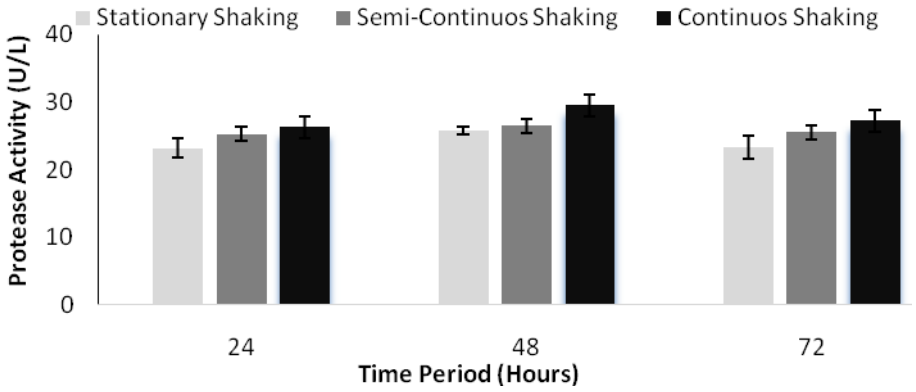

Fig. 1: Optimization of incubation time and fermentation conditions for maximal production of proteases from *Bacillus subtilis*. The error bars represent standard deviation in each case.

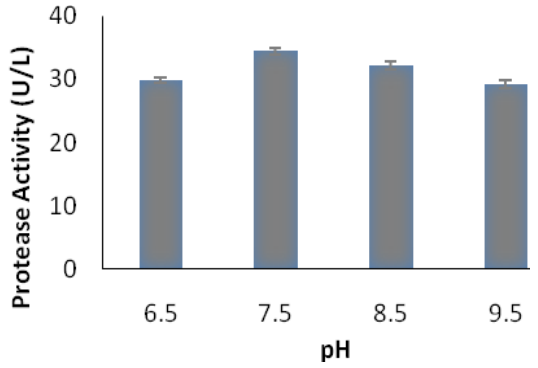

Fig. 2: Optimization of pH for maximal production of proteases from *Bacillus subtilis*. The error bars represent standard deviation in each case.

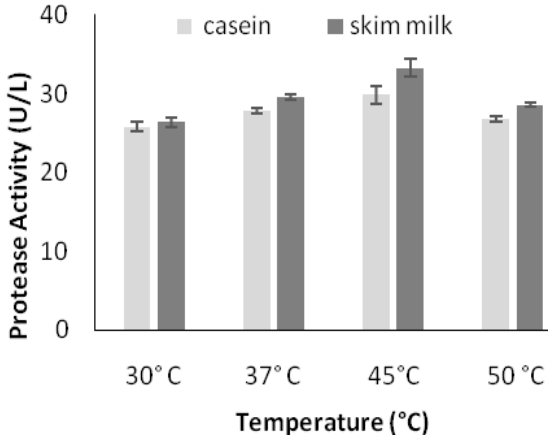

Fig. 3: Optimization of temperature and type of substrate for the maximum production of protease enzyme from *Bacillus subtilis.* The error bars show standard deviation in each case.

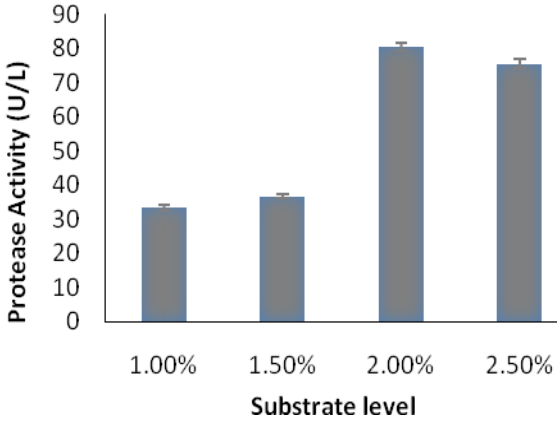

Fig. 4: Optimization of substrate level for the maximum production of protease enzyme from *Bacillus subtilis.* The error bars show standard deviation in each case.

Table 1: Optimization of nutritional biochemicals for maximal production of proteases from *Bacillus subtilis.*

Protease activator	Concentration/ Amount	Mean±SD	Standard Error
Yeast sludge	100µL	133.09±1.66	0.962
	200µL	193.38±1.21	0.701
	300µL	260.48±0.88	0.50
	400µL	134.36±1.26	0.729
Ammonium sulphate	0.2%	247.30±1.03	0.5947
	0.3%	269.55±0.85	0.4929
	0.4%	285.55±1.07	0.6200
	0.5%	279.95±1.19	0.701
Urea	0.1%	206.48±0.84	0.4852
	0.15%	199.39±1.03	0.5958
	0.20%	264.65±0.72	0.4185
	0.25%	250.56±1.23	0.72
Cane molasses	0.03%	220.79±1.76	1.02
	0.05%	154.17±1.53	0.88
	0.08%	161.46±1.23	0.71
	0.10%	179.53±0.94	0.54

Optimization of Temperature and type of substrate

Among the physical factors, temperature is a very critical parameter that could affect bio-processing. To evaluate the same, media inoculated with *Bacillus spp.* was incubated at different temperatures ranging from 30 to 50ºC. It was observed that enzyme production was maximum (33 U/L) at 45ºC (Fig. 3). Low enzyme activity of 28 U/L was noted at 50ºC, (Fig 3). Our results are in accordance with Pant et al. (2015) and Ahmetoglu et al. (2015) who found the maximal production of proteases from *Bacillus sp.* at 45ºC. Different studies reported that enzyme synthesis in bacteria were controlled by temperature and oxygen supply. There is a need to find a suitable temperature that influences maximal secretion of the enzymes (Nassar et al., 2015). They also postulated that a high temperature lowers the growth of *Bacillus sp.* that further decreases the enzyme production.

The substrates used in industrial enzyme fermentations are normally common agricultural products like casein, starch, skim milk and ordinary sugar (Cheetham, 1995). Enzyme production is chiefly dependent on growth of bacterial culture and composition of nutrient medium (Fujiwar et al., 1993). Two substrates casein and skim milk were used in the present study. Results showed that skim milk had better yield as compared to the casein (Fig. 2). The enzymes production was chiefly decreased after 48 h. This might be due to depletion of nitrogen and other sources present in the medium, which were utilized by the microorganisms, or due to inactivation of enzyme by acidification of the medium (Dervoca et al., 1992).

Effect of substrate level

Various substrate levels ranging from 0.5 to 2.5% were evaluated for optimum production of proteases and maximum activity was found at 2% substrate level and

minimum at 0.5% (Fig. 4). Further increase in concentration of substrate may cause depletion of nutrients or autolysis of the enzymes and resulted in less growth of the proteases. It might decrease the aeration and porosity of the medium, which are very essential for proper growth of the enzyme. Same substrate level was also reported by Radha et al. (2011) for the maximal production of proteases from Aspergillus spp. by using submerged fermentation.

Biochemical supplementation

It is essentially required to exploit the positive and negative role of all ingredients (including, ions, molecules and other supplementary sources) being used in enzyme production. It is important to optimize biochemical supplementation levels as elevated level may suppress or inhibit the enzyme production in fermentation. In this study, four supplements were evaluated for their role to produce proteases. Concentrations of four supplementary sources were optimized for maximal production of protease. It was found that the supplements viz yeast sludge, ammonium sulphate, urea and cane molasses had a very positive association to supplement protease production from *B. subtilis* (Table 1). One-way ANOVA showed that the data was highly significant and reliable ($P < 0.05$). Our findings are supplemented by different reported facts. As nitrogen source, yeast sludge and ammonium sulphate were reported to enhance the production of proteases (Lapsongphon et al., 2013; Nilegaonkar et al., 2007). Wang et al. (2005) and Contesini et al. (2017) reported a very vital role of urea and cane molasses to promote the production of proteases. In conclusion, maximum production of proteases from *B. subtilis* was achieved by optimizing various fermentation parameters. The proteases have a significant industrial potential as evident from its optimal pH, temperature and other parameters.

Authors' contribution

All authors contributed equally in this manuscript.

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