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Optimisation of Production Condition of Alkaline Protease Using Indigenous *Bacillus subtilis* from Agricultural Soil

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Abstract. In this study, proteases have been isolated from agricultural soil samples and then cultured by shake flask method. The growth of the *Bacillus subtilis* has been confirmed by microbiological test on the agar plate and skim milk agar in rough, raised and irregular colonies. The yield of the alkaline protease has been optimised by varying the main factors i.e., nitrogen source (peptone, yeast extract, beef extract, casein, ammonium carbonate and urea), carbon source (sucrose, fructose, mannose, lactose, glucose, maltose and starch), incubation period (12, 24, 36, 48, 72, 84 and 96 h), temperature (35, 40, 45, 50, 55, and 60 °C) and salts (potassium sulphate, magnesium sulphate, calcium sulphate and manganese sulphate). The results revealed that the maximum enzyme production was obtained using casein and minimum activity was obtained using urea as a nitrogen source. Similarly, other factors have shown significant effect on the activity of the enzyme.

Keywords: proteases, agricultural soil, shake flask method, optimisation

Introduction

Microbial protease enzymes are widely utilized in most of the industries (Degering *et al*., 2010; Ramnani *et al*., 2005), such as in the detergent, food, pharmaceutical, leather, in peptide synthesis (Horikoshii, 1999), etc. They have a very large and complex group of enzymes with different properties of substrate specificity, active sites and catalytic mechanism, pH, temperature and stability profiles. Proteases have also extensive application in a range of processes due to an advantage of the unique physical and catalytic properties of individual proteolytic enzyme types (Pant *et al*., 2015; Ward, 1991).

Microorganisms are an attractive source of protease as they can be cultured in large quantities in a relatively short time by established fermentation methods producing an abundant, regular supply of the desired product. Besides, they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Gupta *et al*., 2002; Rao *et al*., 1998). Bacterial proteases are mostly extracellular, easily produced in larger amounts, thermostable and active at wider pH range. These properties make the bacterial proteases most suitable for wider industrial applications. Alkaline proteases from bacterial origin are the most important industrial enzymes, which contribute about 60% of the total world enzyme market (Outtrup and Boyce, 1990; Kalisz, 1988; Ward, 1985). These proteases produced by *Bacillus pseudofirmus, Cohnella thermotolerans* and *Bacillus odysseyi* are of great importance in detergent industry due to their high thermo and pH stability (Borsosi *et al*., 2005). This vast diversity of proteases, in contrast to the specificity of their action has attracted worldwide attention in attempt to exploit their physiological and biotechnological applications (Rao *et al*., 1998). The production of extracellular alkaline protease by *Bacillus subtilis* RSKK-96 has been studied with solid state fermentation (Akcan and Uyar, 2011). In the recent years, protease has been produced by *Bacillus licheniformis* cells mobilized on the chitosan, corn cob and corn tassle and production conditions have been optimised (Maghsoodi *et al*., 2013). Another study describes the production of protease by *Bacillus cereus* strain and *Bacillus cohnii* and characterized the enzymatic properties (Lakshmi *et al*., 2014; Tekin *et al*., 2012). Thus, it is needed to search for proteases with significant properties from different sources, and the optimisation of conditions (such as temperature, pH and additives). Therefore, in this study the main objective was to explore the indigenous resources of protease producing bacteria, *Bacillus subitilis* and to determine the optimum conditions of protease production by varying nitrogen source, carbon source, incubation time, temperature, pH and salts.

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Materials and Methods

All chemicals used in this study were analytical grade purchased from Merck and BDH. Shaking Incubator and oven were from BINDER, GmbH, variable speed micro centrifuge was from Centurion Scientific Ltd.

Isolation of bacteria. Twenty five soil samples were collected in polythene bags from four different places of Karachi such as; Korangi industrial area, SITE area, Bin Qasim industrial area and agriculture soil of Memon Goth, where mainly protease were expected as shown in Table 1. A serial dilution (10^2-10^6) of soil samples in sterile double distilled water was prepared and poured into separate 0.5 mL of the culture in petri plates and 15 mL of the melted nutrient agar at 40-50 °C. The plates were mixed thoroughly by gentle rotations and left for solidification. The culture plates were incubated at 37 °C for 24 h and next day number of cells per mL of soil suspended samples were obtained by multiplying the final dilution with numbers of colonies. The selected colonies were used for further study.

Screening for best protease producing strain on medium. The isolates were screened for best protease producing strain by plate assay using protease specific medium containing skim milk, potassium dihydrogen phosphate, glucose, peptone, gelatin and agar-agar, at pH 7.2.

Production of protease. The culture positive for alkaline protease was grown by 100 mL of synthetic medium containing (g/L) nutrient broth with glucose (5 g), peptone (7.5 g), NaCl (5 g), $FeSO_4.7H_2O$(0.1 g), $MgSO_4.7H_2O$(5 g) with the pH 10.0. The medium was incubated for 48 h in shaker incubator (120 rpm) at 37 °C. The fermented broth was filtered and the filtrate was centrifuged at 10,000 rpm for 10 min at 4 °C to extract the crude extracellular protease.

Determination of protease activity by well diffusion method. Agar 1% was prepared using 1% (w/v) casein, 1.5% skimmed milk, and 1% gelatin and poured in petri dishes. The plates were solidified for 30 min and holes of 3 mm diameter were punched. A crude culture supernatant from *Bacillus subtilis* was filled into the holes. These plates were incubated at 37 °C for 24 h. After 24 h of incubation the protease activity was measured by zone.

Determination of the effect of nitrogen sources on alkaline protease production. Alkaline protease production was carried out in mediums 1 to 6 separately along with various nitrogen sources by replacing only peptone with other nitrogen sources like yeast extract, beef extract, casein, ammonium carbonate and urea, respectively. Each nitrogen source was used at 1%(w/v) concentration, respectively.

Determination of the effect of carbon sources on alkaline protease production. Different carbon sources were used in this study such as sucrose, fructose, mannose, lactose, glucose, maltose and starch, to replace the carbon source available in the media. 0.5% (w/v) concentration of different carbon source were used.

Determination of the effects of incubation time, temperature and salt concentration on protease production. The effect of incubation time on protease production was studied by changing incubation duration, such as 12, 24, 36, 48, 60, 72, 84 and 96 h.

The effect of temperature on protease production was studied by maintaining various temperatures like 35, 40, 45, 50, 55 and 60 °C. Incubation time was for 48 h in shaking incubator with 120 rpm. After that, the protease enzyme activity was measured.

The role of salts on alkaline protease production was analysed by the addition of salts like potassium sulphate, magnesium sulphate, calcium sulphate and manganese sulphate added at 0.1 mg/100 mL to the medium.

Results and Discussion

The main sources of the protease enzymes isolation are usually animals (e.g. calf stomach), plants (e.g. pineapple, papaya), and microbes (e.g. *Bacillus* spp., *Pseudomonas* spp.) (Shafe *et al*., 2005; Rao *et al*., 1998), etc. Bacterial proteases are mostly extracellular, easily produced, thermostable and active at wider pH range. Therefore, *Bacillus* was selected to produce protease in this study. The soil samples were collected from different areas of Karachi and subjected to isolation of *Bacillus subtilis* as given in Table 1. The highest abundance of alkaline producing isolates of *Bacillus subtilis* were found in the agriculture soil sample collected from Memon Goth, Karachi whereas no isolates were found in the sample collected from Bin Qasim industrial area, Karachi. These isolates were characterized by their alkaline pH (8-12), with optimal temperature between 35 °C and 60 °C. The growth of *B. subtilis* was confirmed by microbiological test on the nutrient agar plate and skim milk agar. The colonies on the nutrient agar plates were rough, raised, and irregular with rough margins, while

on the skim milk agar, the colonies were glossy, smooth with rounded margins and clear zone of proteolysis. *Bacillus* was derived alkaline proteases and stable at elevated temperatures and pH, but majority of them were incompatible with detergent matrices as reported earlier by Deng *et al*. (2010).

Proteases are differing in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature and stability profiles. Studies relating to such properties are essential for the successful application of these enzymes in their respective industry (Sumantha *et al*., 2005). Among the organic nitrogen sources peptone, yeast extract, beef extract, casein, ammonium carbonate and urea had significant effect on protease production 560U/mL, 535U/mL, 590U/mL, 650U/mL, 468U/mL, 421U/mL, respectively. It was observed that the alkaline protease production was good in all cases but maximum enzyme production was obtained using casein and minimum was obtained using urea as nitrogen source as shown in Table 2, and agreed with results of Haddar *et al*. (2009). The amount of protease produced from microorganisms depends on the nature of strains and the composition of the growth medium (Nadeem *et al*., 2007; 2006). Actually, protease are produced at exponential phase of bacterial growth which is associated with the sporulation of *B. subtilis* (Elliot and May, 1968), whereas casein is a source of amino acids that enhances the foam formation to remove spores and cellular debris from the culture.

The effect of change of carbon source was determined by measuring enzyme activity. Different sources of carbon such as sucrose, fructose, mannose, lactose, maltose, and starch were used and replaced with the glucose as a carbon source in growth media. Results showed that the enzyme activity was highest in the glucose as compared to other carbon sources at 24 h and 48 h of incubation. Sucrose and fructose also showed high protease activity at 24 h, but extremely reduced by 48 h of incubation as shown in Table 3. This observation also supports the earlier reports which suggested that sources of carbon affected production of enzymes by bacteria (Juhasz *et al*., 2003). At 72 h incubation due to the prolonged incubation time perhaps led to auto digestion of proteases and proteolytic attack by other proteases (Horikoshii *et al*., 2006). Incubation time plays a substantial role in maximizing bacterial growth and protease production. Results in Table 4 showed that the enzyme activity was different at different incubation periods. It was maximum at 72 h, while

Table 1. Sample collection and isolation of bacterial cells/mL

Area of collection	No. of samples	No. of isolates	No. of cells/mL
Korangi industrial area	5	4	38000
Site area	8	3	33000
Bin Qasim industrial area	4	0	00
Agriculture soil (Memon Goth) Karachi	8	1	43000

Table 2. Effect of nitrogen sources on protease enzyme activity

Bacterial specie	Nitrogen sources	Enzyme activity (U/mL)
Bacillus subtilis	Peptone	560 ± 2.87
	Yeast extract	535 ± 2.11
	Beef extract	590 ± 2.33
	Casein	650 ± 3.20
	Ammonium carbonate	468 ± 2.35
	Urea	421 ± 3.34

Table 3. Effect of carbon source on the protease enzyme activity

Bacterial specie	Carbon source	Enzyme activity (U/mL)
Bacillus subtilis	Sucrose	620 ± 2.50
	Fructose	580 ± 2.73
	Mannose	320 ± 1.57
	Lactose	292 ± 1.75
	Glucose	670 ± 3.20
	Maltose	340 ± 2.33
	Starch	280 ± 2.15

Table 4. Effect of incubation periods on protease enzyme activity

Bacterial specie	Incubation period (h)	Enzyme activity (U/mL)
Bacillus subtlis	12	232 ± 1.53
	24	290 ± 1.73
	36	455 ± 1.57
	48	538 ± 1.66
	72	623 ± 2.20
	84	559 ± 2.31
	96	520 ± 2.44

lowest at 12 h. Temperature is also one of the most critical parameters that has to be controlled in bio-processing (Chi *et al*., 2007). Therefore, the effect was optimised at different temperatures. The enzyme activity was highest at 45 °C and lowest at 60 °C (Table 5).

An increase in the protease activity was also observed in the presence of Mg^{+2} as shown in Table 6. It was also studied earlier that the metal ions such as; Ca^{+2}, Mg^{+2} and Mn^{+2} increase the enzyme stabilization which results in the increase in enzyme activity and thermal stability of alkaline proteases at higher temperature (Kumar, 2002). Further, biochemical characterisation and application of enzyme is in progress.

Table 5. Effect of temperature on protease enzyme activity

Bacteria specie	Temperature (°C)	Enzyme activity (U/mL)
Bacillus subtilis	35	356 ± 2.66
	40	545 ± 1.57
	45	635 ± 2.20
	50	510 ± 1.66
	55	412 ± 1.25
	60	210 ± 2.11

Table 6. Effect of salts on protease enzyme activity

Bacteria specie	Salts	Enzyme activity (U/mL)
Bacillus subtilis	Potassium sulphate	432 ± 2.77
	Magnesium sulphate	620 ± 3.55
	Calcium sulphate	300 ± 3.66
	Manganese sulphate	358 ± 2.25

Conclusion

Bacillus subtilis is a good source for the production of protease enzymes which have many uses in industries. It is concluded in this study that the optimisation of protease production from *Bacillus subtilis* had led for better resource and appropriate factors such as; the agricultural soil was found a better source of micro-organisms, casein as a best nitrogen source, glucose as a best carbon source, 48 h as best incubation period and 45 °C as a best temperature for *Bacillus subtilis* growth.

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