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PARTIAL PURIFICATION AND CHARACTERIZATION OF AN ALKALINE PROTEASE FROM *ASPERGILLUS TERREUS* USING CORN HUSK AS A NOVEL SUBSTRATE.

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Abstract

Corn husks are the dried outer sheaths that surround each ear of corn. It contains high cellulose content and protein content in some extent and act as a carbon and nitrogen source for protease production. In the present investigation protease production by *A. terreus* on corn husk was studied. Corn husks moistened with D3 diluent showed maximum protease production (90 U/g dry solids) when fermented by *A. terreus.* Maximum Protease production was obtained at 40ºC temperature, 9.5 pH, and at 3rd day of incubation. Purification increased its specific activity 4.2 -fold with a yield of 61% .Partially purified protease showed maximal activity at a temperature of 40 °C and pH of 9.0.

Index terms- Alkaline Protease, *Aspergillus terreus*, Corn husk, SSF.

Introduction

Proteases are proteolytic enzymes, which hydrolyze the peptide bonds present in proteins to convert it into shorter polypeptides and amino acids. Protease is one of the most important commercial enzymes, and is used in food processing, detergents, dairy industry and leather making industries (Negi and Banerjee, 2010).

Microbial proteases are predominantly extracellular and can be secreted in the fermentation medium. The proteases of *Aspergillus* species, in particular, have been studied in detail since they are known for their capacity to secrete high levels of enzymes in their growth environment. Fungal alkaline proteases are generally produced by both submerged and solid-state fermentation (Devi et al., 2008, Sindhu et al., 2009, Impoolsup et al., 1981, Chutmanop et al., 2008, Anandan et al., 2007, Pandey et al., 1999) also describes the tremendous potential of SSF for the production of various enzymes of industrial importance and their direct agro-biotechnological applications. From commercial point of view SSF offers many advantages including superior volumetric productivity, use of cheaper substrates, simple downstream processing, use of simpler machinery and lower energy requirements when compared with submerged fermentation (Paranthaman et al., 2009).

Corn husks are the thin cellulose-rich leafy sheaths that cover the corn cobs (Mahalaxmi et al., 2010). It contains high cellulose content and in some extent protein content is also present. Due to its availability, biodegradable nature, easy release of carbon sources and high water holding capacity, the role of corn husk as the solid substrate for protease production by isolated *Aspergillus terreus* was investigated in the present study.

MATERIAL AND METHODS

Microorganism and maintenance of culture - *Aspergillus terreus* used in this study was isolated from soil samples in Jabalpur area and was identified on the basis of molecular analysis by Agharkar Research Institute, Pune (MH). The fungus was grown and maintained on potato dextrose agar (PDA) slants at 28±2°C with fortnightly transfer to fresh medium.

Substrate – Corn Husk used in this study was obtained from local market in Jabalpur, M.P. India.

Inoculum preparation -The inoculum was prepared by dispersing the spores from a week old fungal slant culture in 0.1% Tween- 80 solution with a sterile inoculating loop.

Solid state fermentation -Five grams of Corn Husk was taken in a 250 ml Erlenmeyer flask moistened with 5 ml of diluent, sterilized at 121°C for 15 min, cooled inoculated with 1 ml of fungal spore suspension and incubated at 28±20°C for 96 h unless otherwise specified .

Influence of diluents on protease production following eight diluents (pH adjusted to 8.5) were used to moistened the substrate [Ikram and Sikandar 2003].

D1: (%W/V) Peptone, 1.0 dextrose 4.0

D2: (%W/V) $NaNO_3$, 2.0 ; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.05; $CaCl_2.2H_2O$, 0.05; $ZnSO_4.7H_2O$, 0.01

D3: (%W/V) Glucose, 7.0 ; Peptone, 2.0 ; KH_2PO_4, 0.4; $MgSO_4$. $7H_2O$, 0.05; $CaCl_2.2H_2O$, 0.05; $ZnSO_4$. H_2O, 0.01

D4: (%W/V) Yeast extract, 1.0; Glucose, 3.3; Peptone, 1.0; $CaCO_3$, 1.0

D5: (%W/V) Glucose, 1.0; Peptone, 1.0 ; Beef extract, 1.5 ; NaCl, 0.3

D6: (%W/V) Glucose, 2.0; Peptone, 1.0 ; Beef extract, 1.0; NaCl, 0.3; $CaCl_2$, 0.1 ; Na_2CO_3, 0.7

D7: (%W/V) $(NH_4)_2$ SO4, 1.4; Urea, 3.0 KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 3.0; $CaCl_2$, 3.0

D8: Distilled Water.

Optimization of physical parameters- Optimization of growth conditions and enzyme production was done using different parameters such as incubation time, pH and temperature.

Incubation time - The effect of incubation period on enzyme production (pH 8.5; temperature 28±20C) was investigated by checking the enzyme activity on 0, 24 h, 48h, 72h, 96h, 120h, 144h and 168 h of incubation.

pH - The effect of pH on enzyme production was investigated by SSF in Corn Husk by adjusting the pH of D3 diluent as the maximum enzyme production was obtained when this diluent was used as moistening agent. pH of the diluent was adjusted to 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5 and 11.0 using 1 M NaOH. The substrates inoculated with *A. terreus* were then incubated for 72 h at 28±2°C as the maximum enzyme production was at the 72 h of incubation.

Temperature - The favorable temperature for maximum protease production was studied with a range of 10°C to 65°C. Substrate was moistened with D3 diluent (pH-9.5) and incubated for 72 h as the maximum enzyme production was obtained under these conditions.

Crude enzyme extraction - A known quantity (5g) of the fermented material was mixed with 10ml of Tween 80 (0.1%) solution in distilled water and homogenized by mixing in a rotary shakes at 150 rpm for 1h. The solids were removed by filtration and filtrates were centrifuged at 8000 rpm for 15 mins at 40°C. The resultant supernatant was used for analytical studies.

Assay for protease activity - Protease activity was assayed by the method of Anson [1938].

Partial purification of protease: All operations were performed at 4° C. The enzyme was precipitated from crude extract by the gradual addition of solid ammonium sulphate with gentle stirring to 75% saturation and the pellet was collected by centrifugation at 10,000 rpm for 10 min. The protein pellet obtained after saturation was dissolved in 0.1M Tris- HCl buffer, and loaded on to prepacked desalting column of cross linked dextran with epichlorohydrin, equilibrated with Tris-HCl buffer, pH 7.8. The protease was eluted at 1.0M NaCl concentration.

Effect of pH and temperature on desalted protease activity: The optimum pH for activity of the purified protease was investigated by measurements at 37° C in buffers of various pH. The pH was adjusted using the following buffers – phosphate buffer (pH 7.0); Tris HCl buffer (pH 8.0), glycine – NaOH buffer (pH 9.0- 11.0). The effect of temperature on its activity was investigated keeping pH 7.5 constant. Protease activities were assayed at standard assay conditions.

RESULTS AND DISCUSSION

Influence of various diluents on protease production –

Influence of various diluents on the production of protease by *A.terreus* grown on corn husk as a substrate was studied. In the present investigation the maximum enzyme production of 110U/g of dry solids was observed when the substrate was moistened with D3 diluent while minimum protease production was (42U/g of dry solids) obtained when moistened with D8 diluent i.e. distilled water (Figure -1). Similar result was observed by Sharma N. (2012) when alkaline protease was produced by *A. terreus* on mustard oil cake. The ratio of substrate to diluent was kept 1:1. When substrate was moistened with glucose, peptone, $CaCl_2$, $MgSO_4$, KH_2PO_4 (D3 diluent components), enzyme synthesis was maximum. It shows that organic nutrients (glucose, peptone) and inorganic nutrients (CaCl2, ZnSO4, and MgSO4) both stimulate the production of enzyme from *A. terreus*. Potassium dihydrogen phosphate in this diluent act as a constituent of the extraction buffer and may enhanced protease recovery from corn husk.

Figure 1- Influence of diluents on protease production

Table-1 - Impact of Various diluents on protease production by *Aspergillus terreus* in SSF using Corn Husk as a substrate.

Diluents	Protease activity (U/g)
D1	147.4
D2	156.2
D3	171.6
D4	151.8
D5	143
D6	147.4
D7	134.2
DW	101.2

Optimization of physical parameters

Protease production in fungal cultures is growth associated and is influenced by various factors and their interaction can affect protease productivity. Physical parameters like pH and temperature of the fermentation medium play a vital role in the growth and metabolic production of fungal population. The pH of the fermentation medium is reported to have substantial effect on the production of proteases (Yadav et al., 2011, Sindhu et al., 2009, Hajji et al., 2008, Hajji et al., 2007). Fermentation time also has an impact on enzyme production.

Incubation time- The time of incubation has an important role in cheaper commercial production of enzymes. Fig: 1and Table -2 show that *A. terreus* gave the maximum yield of protease at 72 h of incubation in a SSF process with Corn husk as the substrate. The subsequent decrease in enzymes activity with increasing fermentation time could possibly be due to cessation of production by SSF [Pandey A 2003].

Table-2 - Impact of incubation time on protease production by *Aspergillus terreus* in SSF using Corn Husk as a substrate.

Incubation time (In hrs)	Protease activity (U/g)
24h	67.1
48h	90.2
72h	120.0
96h	69.3
120h	77.0
144h	89.1
168h	83.6
194h	78.1

pH - The influence of initial pH on production of protease by *A.terreus* was determined in the pH range of 7- 11. Protease synthesis increased with increase of initial pH of the substrate and reached maximum at pH 9.5; whereas below and above this level of pH yield was low (Table:3). The pH dependent changes in the amount of enzyme production might have been due to pH dependent control of protease gene expression. Similar studies on the influence of pH on protease production have been studied in different *Aspergillus* sp. In case of *A.terreus* the optimum pH was reported to be between 5.5 and 9.5 [Chakrabarthi et al., 2000].

Table3 - Impact of incubation pH on protease production by *Aspergillus terreus* in SSF using Corn Husk as a substrate.

Incubation pH	Protease activity (U/g)
7.0	34.1
8.0	46.2
8.5	61.6
9.0	75.9
9.5	82.5
10.0	51.7
11.0	44.0

Incubation temperature - SSF is more sensitive to temperature variation than broth culture, not only does the temperature regulate the synthesis of the enzyme but possibly the secretion of the enzyme by changing the properties of the cell wall [Satyanarayana J1994]. The maximum protease activity was recorded at 40°C. An optimum temperature of 40°C for *A.terreus* has also been reported which is similar to our study and Niyonzima and More (2013) reported optimum temperature of 37 C under Smf by *A.terreus*. Inactivation at higher temperatures may be attributed to incorrect conformation due to hydrolysis of the peptide chain, disruption of amino acids or aggregation.

Table-4 Impact of incubation temperature on protease production by *Aspergillus terreus* in SSF using Corn Husk as a substrate.

Incubation temperature(°C)	Protease activity (U/g)
10	27.5
20	29.7
30	38.5
40	55.0
50	47.3
60	44.0
65	41.8

Partial purification of protease

Table -5- shows the different steps of purification for *Aspergillus terreus* with 61% yield and 4.2 fold purification.

Fraction	Total Activity U	Total protein (mg)	Specific Activity (U/mg)	Fold purification	Overall Yield(%)
Crude extract	4839	14	345.6	1	100
Ammonium sulphate precipitation and gel filtration (desalted)	1735	1.2	1446	4.2	61

Effect of pH and temperature on desalted protease activity

This alkaline protease from *Aspergillus terreus* was characterized. The optimum pH was found to be 9.0 (Table-6), indicating that this enzyme might be the alkaline protease. Similar result are observed in the protease produced by *Aspergillus oryzae* AWT 20 sp.(Sharma et al., 2006), *Bacillus circulans* (Jaswal and Kocher, 2007) and *Aspergillus clavatus* (Celia et al., 2007). The optimum temperature for protease activity was determined by screening temperatures from 20° to 75° C using casein as a substrate dissolved in phosphate buffer at pH 7.5. The protease showed best activity at 40° C but was inactive beyond 65° C temperature. Thus optimum temperature of protease activity was 40° C (Fig: 1). This result also confirmed that the enzyme produced by this *Aspergillus terreus* was an alkaline protease.

Table-6

Incubation pH	Protease activity (U/ml)	Incubation temperature(°C)	Protease activity (U/ml)
7.0	84.4	10	81.0
8.0	154.0	20	83.5
9.0	177.6	30	87.3
10.0	148.5	40	139.7
11.0	125.0	55	104.5
-	-	65	99.0
-	-	75	81.5

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