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RESEARCH ARTICLE

Optimization of protease production by *Bacillus sp*. MPTK 712 under solid state fermentation using green gram husk

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ABSTRACT

This study evaluated the feasibility of easily available substrates in solid state fermentation (SSF) for the production of protease by *Bacillus sp.* MPTK 712. The production parameters were optimized against incubation period, moisture content, initial pH, extraction medium, various carbons, nitrogen, and metal salts sources and optimum temperature of enzyme activity. It was found that green gram husk, 96 h, 30% moisture, 50°C, arabinose, beef extract and ferrous sulphate influenced protease production. Considerable increase in protease production was noticed after the optimization process. The optimized media was subjected to purification process which yielded a specific activity of 3506 U mg^{-1} after Q-sepharose chromatography.

INTRODUCTION

Proteases are one of the most important commercial enzymes constituting 60-65% of the global enzyme market (Chi *et al.,* 2007). They are widely used in food processing, detergents, diary industry and leather making (Priyanka *et al.,* 2010). Proteases are produced by a wide range of microorganisms including bacteria, moulds and yeasts. In bacteria, this enzyme is produced mainly by many members belonging to genus *Bacillus* especially, *B. licheniformis, B. horikoshii, B. sphaericus, B. furmis, B. alcalophilus, B. subtilis* (Ellaiah *et al*., 2002). At present, the overall cost of enzyme production is very high (due to high cost of substrate and medium used) and therefore, development of novel processes to increase the yield of proteases with increasing the production cost is highly appreciable from the commercial point of view. To achieve these goals, during the recent years, effects have been directed to expose the means to reduce the protease production costs through improving the yield, and the use of either cost free of low cost feed stocks or agricultural by products as substrates for protease production (Kuberan *et al*., 2010). In recent years, solid-state fermentation (SSF) involving growth of microbes on moist solid substrate(s) in the absence of free flowing water, has gained a tremendous momentum owing to certain advantages over the conventional submerged fermentation (SmF), like low production cost, saving of water and energy, less waste effluent problem and stability of the product due to less dilution in the medium (Pandey, 2003, Holker and Lenz, 2005). Therefore, SSF has found several potential applications in the industrial production of value added products, such as industrially important microbial enzymes, bioinsecticides, secondary metabolites and pharmaceuticals (Robinson *et al*., 2001, Pandey, 2003). This study was aimed to evaluate the feasibility of easily available substrates in SSF for the production of protease by *Bacillus sp.* MPTK 712. The production parameters were optimized against incubation period, moisture content, initial pH, extraction medium, various carbons, nitrogen, and metal salts sources and optimum temperature of enzyme activity. After the optimization process, protease was purified from the production medium by chromatography and its molecular weight was determined by SDS-PAGE.

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MATERIALS AND METHODS

Bacterial strain

The test isolate *Bacillus sp.* MPTK 712 was isolated from Aavin industry dairy effluent located at Shozhlinganallur, Chennai, Tamil Nadu.

Substrates

The substrate used in this study namely Rice bran, Wheat bran, Soya bean meal, Ground nut cake, Green gram husk and Coconut oil cake was obtained from a local market in Chennai.

Solid state fermentation

In an attempt to choose a potential substrate for SSF which supports protease production, various substrates namely Rice bran, Wheat bran, Soya bean meal, Ground nut cake, Green gram husk and Coconut oil cake were screened individually. SSF was carried out by taking 3 g of dry substrate in a 100 mL Erlenmeyer flask to which distilled water was added to adjust the required moisture level. The contents of the flasks were mixed and autoclaved at 121°C for 15 min. Flasks with inoculated were shaken at 150 rpm at 37°C for 144 h. The contents of the flasks were harvested and assayed every 24 h.

Enzyme extraction

The enzyme from the fermented media was extracted twice with tap water. The slurry was squeezed through damp cheesecloth. Extracts were pooled and centrifuged at 4°C for 15 min at 10,000 rpm to separate small particles of different substrates, cells and spores. The brown, clear supernatant was used in enzyme assays.

Protease assay

Protease activity was determined using sulphanilamide azocasein according to the method of Leighton *et al*. (1973). The reaction mixture containing 250 µL 1% (w/v) substrate in 0.1M Tris/HCl buffer (pH 9.0) and 150 µl of enzyme solution was incubated for 30 min at 37°C. After incubation, the enzyme was inactivated by addition of 1.2 mL trichloroacetic acid solution (10%, v/v) and then the solution was neutralized using 800 µL of 1.8 N NaOH solution. The absorbance was read at 420 nm. One unit of proteolytic enzyme activity was defined as the amount of azocasein that hydrolyzed during 30 min incubation at 37°C for millilitre of solution of extract. All experiments were conducted in triplicate.

Effect of moisture content

To check the influence of moisture on protease activity during SSF, Green gram husk was moistened with different amounts of distilled water (20%, 30%, 40%, 50%, 60% and 70%) prior to fermentation.

Effect of incubation time, pH and temperature

The effect of incubation time (24-144 h) was studied in different time intervals with the optimized pH and temperature of the production medium inoculated with the test isolate. The production medium with the optimized substrate was adjusted at various levels of pH by NaOH solution (4, 5, 6, 7, 8, 9, 10 and 11) and the effect of initial pH on protease production was studied. To study the effect of incubation temperature on protease production, the flasks with the production medium were inoculated and incubated at various temperatures ranging from 35-65°C for 24 h.

Effect of carbon and nitrogen sources

Mannose, xylose, lactose, sucrose, fructose, galactose, glucose, and arabinose were used as co-carbon sources at 1% along with the optimized substrate. The carbon sources were inoculated with the MPTK 712 strain for enzyme production. Organic and inorganic nitrogen sources like Beef extract, Casein, Yeast extract, Peptone, Urea, NH_4Cl, $(NH_4)_2SO_4$ and N_aNO_3 were amended to the production medium at 1% along with the test isolate and optimized substrate for enzyme production.

Effect of metal ions

The effect of several metal ions ($FeSO_4$, $MnSO_4$, $CuSO_4$, $ZnSO_4$ and $MgCl_2$) on the enzyme production of the isolate was determined. The enzyme solution was stored for 1 h at 30°C in the presence of 1 mM of various ions prior to the respective enzyme assays for remaining activity.

Purification of protease

The crude extract of the fermentation medium was subjected to 85% ammonium sulphate precipitation by continuous stirring. The saturated solution was kept undisturbed overnight at 4°C. It was then centrifuged and the precipitate was dissolved in a small amount of 50 mM glycine–NaOH buffer (pH 10.5) and dialyzed for 24 h. Two ml of dialyzed protein was loaded on Q-Sepharose column, (1.5 cm X 12 cm) pre-equilibrated with glycine–NaOH buffer. The flow rate was adjusted to 30 ml/h and 2 ml fractions were collected and analyzed for the protease activity. The fractions with maximum activity were pooled, concentrated, and then used as source of protease for further characterization of enzyme. Total amount of protein throughout the experiment was measured according the method of Lowry *et al.* (1951) using Bovine serum albumin (BSA) as standard. The purity of the alkaline protease was checked using 10% denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The polyacrylamide gel was prepared by the method of Laemmli (1970). The staining was done with Coomassive brilliant blue staining.

RESULTS AND DISCUSSION

The overall cost of protease production is very high due to high cost of substrates and mediums used and therefore, development of novel processes to increase the yield of proteases with respect to their industrial requirements coupled with lowering down the production cost is highly appreciable from the commercial point of view. To achieve these goals, during the recent years, efforts have been directed to explore the means to reduce the protease production costs through improving the yield, and the use of either cost-free or low-cost feed stocks or agricultural by-products as substrate(s) for protease production (Sandhya *et al*., 2005). Over the past couple of years, solid-state fermentation (SSF) involving growth of microbes on moist solid substrate(s) in the absence of free flowing water, has gained a tremendous momentum owing to certain advantages over the conventional submerged fermentation (SmF) (Pandey, 2003, Holker and Lenz, 2005). Different natural substrates namely Rice bran, Wheat bran, Soya bean meal, Ground nut cake, Green gram husk and

Coconut oil cake were screened for protease production by SSF. It was found that green gram husk recorded highest protease activity (252 U/ml) after 96 h of incubation (Fig. 1). Since green gram husk produced highest protease activity, further optimization of process parameters was done using this natural substrate.

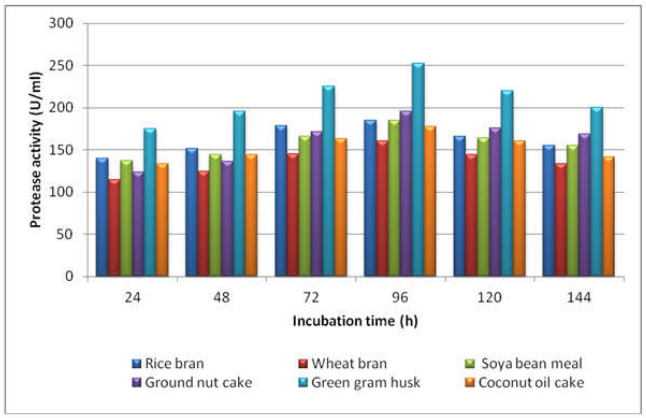

Fig. 1. Screening of best substrate for protease production by SSF

It is very essential to detect the optimum incubation time at which an organism exhibit highest enzyme activity since organisms show considerable variation at different incubation periods. It was noted that a high protease activity (252 U/ml) by the test isolate with green gram husk was obtained at 96 h of incubation time (Fig. 2). The decline in enzyme activity observed after 120 h of incubation might be due to denaturation and/or decomposition of protease as a result of interactions with other compounds in the fermented medium (Uyar and Baysal, 2004). The findings of the study fall in line with others on *Bacillus sp*. (Studdert *et al.*, 1997; Ferreri *et al.*, 1996; Takii *et al.*, 1990). Protease production nearly corresponded maximum in early stationary phase (Kuberan *et al.*, 2010).

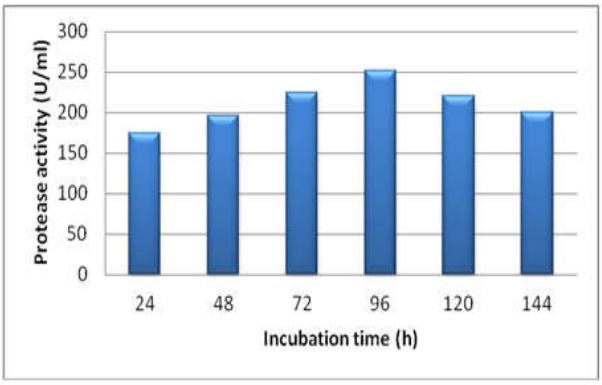

Fig. 2. Effect of incubation time on protease production by *Bacillus sp*. MPTK 712 with green gram husk as substrate

The initial moisture content significantly influence hydrolytic enzyme production in SSF. This is important for microbial growth and enzyme production under solid state fermentation using a particular substrate, moisture level (content)/water activity is one of the most critical factors. Because, SSF processes are different from submerged fermentation culturing, since microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents (Pandey *et al.*, 2000). It was found that 30% moisture content facilitated highest protease activity by the test strain (Fig. 3).

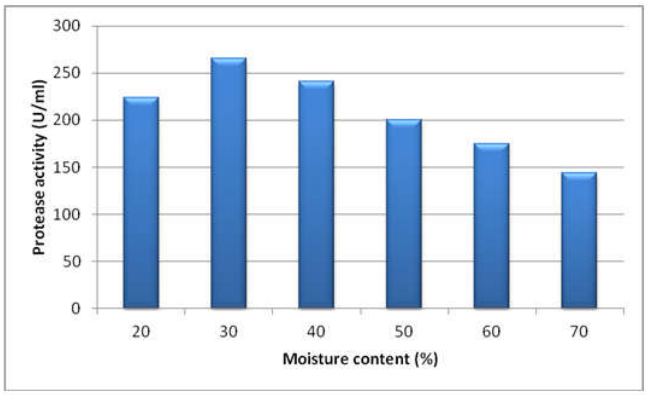

Fig. 3. Effect of moisture content on protease production by *Bacillus sp*. MPTK 712

The pH is an important factor which affects the growth and enzyme production during solid-state fermentation (Ellaiah *et al.*, 2002). From figure 4 it was noted that an alkaline pH 9 favoured maximum lipase production (278 U/ml). The enzyme activity decreased after pH 9 indicating the test strain was alkalophilic in nature. This report was in accordance with the findings of Akcan and Uyar (2011) who found the optimum pH of 9 for protease activity by *Bacillus sp*. in a lentil husk supplemented SSF.

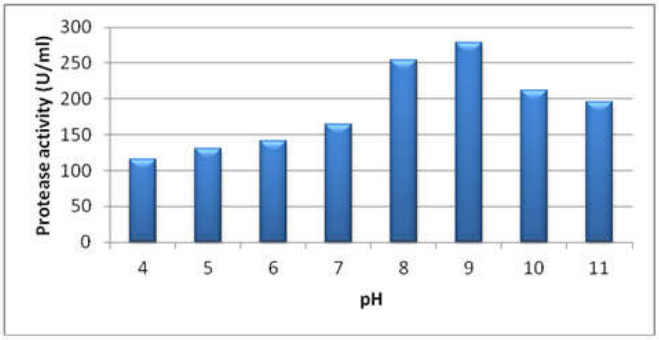

Fig. 4. Effect of pH on protease production by *Bacillus sp*. MPTK 712

Incubation temperature plays an important role in SSF. To study the effect of temperature on enzyme production, the SSF was carried out at different temperatures 35-65°C. It was noted that a high temperature about 50°C exhibited highest protease activity (285 U/ml) indicating the thermotolerant nature of the enzyme (Fig. 5).

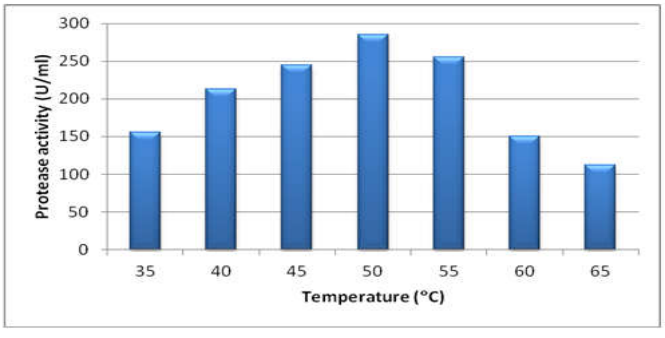

Fig. 5. Effect of temperature on protease production by *Bacillus sp*. MPTK 712

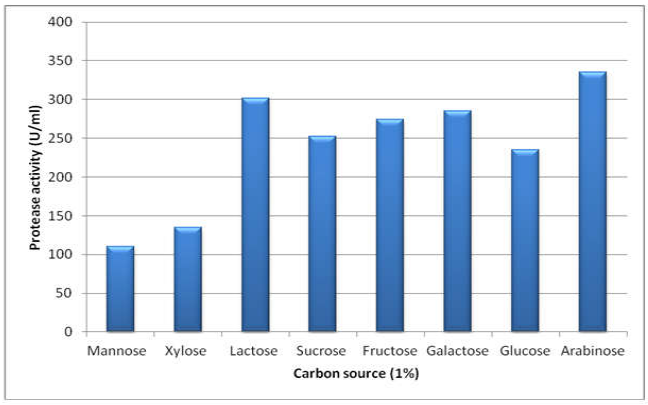

Fig. 6. Effect of carbon source on protease production by *Bacillus sp.* MPTK 712

Among the various carbon sources tested, arabinose was found to be the best support protease production with a concentration of 1% (Fig. 6). In addition, good protease activity was also observed in the media supplemented with lactose, galactose, and fructose. Carbon sources like mannose and xylose recorded low protease activity. This may be due to the total repression of enzyme synthesis described a complete inhibition of the extracellular protease production (Joo *et al*., 2002).

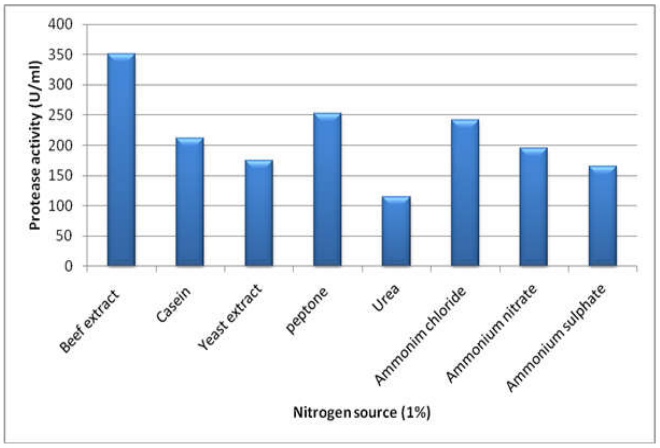

Fig. 7. Effect of nitrogen source on protease production by *Bacillus sp*. MPTK 712

The culture environment has a dramatic influence on enzyme production especially nitrogen sources play a crucial role in enzyme induction in bacteria (Elibol and Ozer, 2001). The effect of various organic and inorganic nitrogen sources on protease production is shown in figure 7. Beef extract recorded maximum protease production compared to all other nitrogen sources employed. It has been reported that beef extract increased protease production by *B. subtilis* DM-04 (Mukherjee *et al*. 2008). Yang *et al*. (2000) found that protease production by *B. subtilis* Y-108 was repressed by most of the nitrogen sources employed by SSF. Effect of metal ions on protease production by *Bacillus sp*. MPTK 712 was investigated. It was found that $FeSO_4$ and $MnSO_4$ influenced protease production (Fig. 8). The addition of $ZnSO_4$ repressed protease production by *Bacillus sp*. MPTK 712 there are various reports recording positive effects of metal cations on protease production (Wang *et al*., 2005; Haddar *et al*., 2009; Uyar *et al*., 2011).

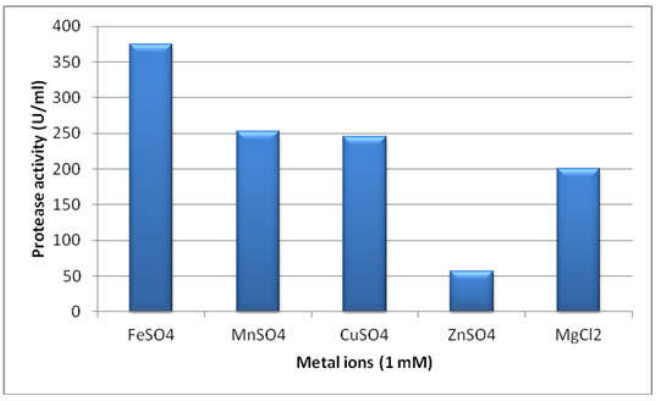

Fig. 8. Effect of metal ion protease production by *Bacillus sp*. MPTK 712

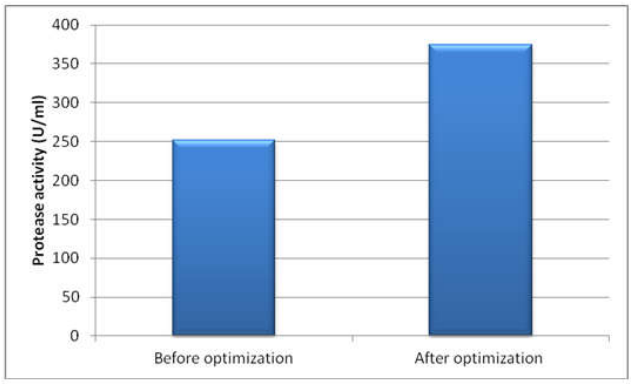

Fig. 9. Protease activity recorded before and after enzyme optimization

There was a considerable and high increase in the enzyme activity depicted by *Bacillus sp*. MPTK 712 after the SSF process. The test isolate recorded an enzyme activity of 252 U/ml before the SSF process. The enzyme activity rose to 375 U/ml after the SSF process (Fig. 9). Thus, this optimized fermentation medium was further employed for the purification process. In SSF process, the crude extracts contain different mixtures of proteins and undesirable products as organic acids and other metabolites. So that purification of the required favourable product must be take place by different purification methods. In most enzyme purification the precipitation of the crude total proteins after separating the culture from the fermentation broth by filtration or centrifugation and the culture supernatant is concentrated by salting out by solid ammonium sulphate as a precipitation agent (Kumar *et al.,* 2002) and followed by another purification procedure as ion exchange and gel filtration chromatography. This study used ammonium sulphate as a precipitation agent recording 1141 U mg^{-1} of specific activity and 3506 U mg^{-1} after the Q-sepharose chromatography (Table 1).

Table 1. Purification scheme of protease from Bacillus sp. MPTK 712 by SSF

Steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})
Crude enzyme	2200	856000	390
Ammonium sulphate perception (85%)	540	616000	1141
Q-Sepharose	87	305000	3506

Many researchers used SDS-PAGE to detect the homogeneity and molecular weight of alkaline proteases produced by bacteria and actinomycetes (Rao and Narasu, 2007). The homogeneity of the purified protease from *Bacillus sp.* MPTK 712 was detected by using SDS-polyacrylamide gel and showed that one only band within the gel electrophoresis (Fig. 10), which indicate that the purified protease is homogenous, also have a molecular weight equal to 26,000 Daltons.

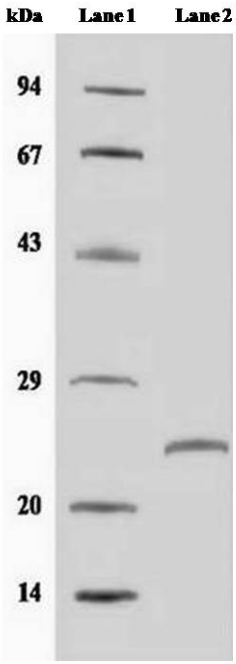

Fig. 10. Photographic representation of SDS-PAGE Gel (Lane 1, Marker Proteins; Lane 2 showing Protease single band after Q-Sepharose)

Conclusion

This study evaluated the feasibility of easily available substrates in SSF for the production of protease by *Bacillus sp.*MPTK 712.The production parameters were optimized against incubation period, moisture level, initial pH, extraction medium, various carbon, nitrogen, and metal salts sources and optimum temperature of enzyme activity. It was found that green gram husk, 96 h, 30% moisture, 50°C, Arabinose, Beef extract and ferrous sulphate influenced protease production. Considerable increase in protease production was noticed after the optimization process. The optimized media was subjected to purification process which yielded a specific activity of 3506 U mg^{-1} after Q-sepharose chromatography. To conclude, this study confirmed that cultural environment plays a vital role in protease production by *Bacillus sp*. MPTK 712 under SSF. The purified enzyme may be further exploited towards various industrial applications in near future.

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