Development of Bioprocess for Improved Production of Alkaline Protease by Mutant Strain of Aspergilllus flavus in Solid State Fermentation using Agricultural Wastes

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Citation

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Abstract

A mutant of Aspergillus flavus MTCC 9952, developed by chemical mutagenesis, produced 37% higher alkaline protease over wild type after optimization of fermentation conditions. The agro-industrial wastes wheat bran and corn cob in the ratio of 1:1 with initial moisture content of 50% were found to be most suitable substrates for protease production under solid state fermentation. Maximum alkaline protease was produced when culture was incubated at 37°C with initial medium pH 9.0 in 48 h with inoculum size of 1x108 spores/gram IDS. Supplementation of fructose (200 mg/g IDS) as carbon and malt extract (100 mg/g IDS) as nitrogen source with other ingredients like di-ammonium hydrogen phosphate (20 g/g IDS) and CuSO4 (0.5 mg/g IDS) considerably increased the protease production. An inexpensive and readily available agro by-product can be used for production of industrially important enzymes.

INTRODUCTION

Proteases are important group of enzymes from an industrial perspective and cater for the requirement of nearly 60% of the world enzyme market (Kalisz, 1998). Alkaline proteases execute a large variety of complex physiological, metabolic and regulatory functions which is evident from its occurrence in all forms of living organisms (Wandersman, 1989). Furthermore, these enzymes are of great interest from a biotechnological angle and are being investigated not only in scientific areas like protein chemistry and protein engineering, but also find application in detergent, food, pharmaceutical and tannery industries (Kumar and Takagi, 1999).

In the last few decades, the exponential increase in the application of proteases in various fields instigated the research in both qualitative improvement and quantitative enhancement of proteases. To achieve commercially viable production levels, it requires strain-improvement programs as the production level of the enzymes in naturally occurring strains is, in most cases, too low for commercial exploitation (Verdoes et al., 1995). In recent years the genetic engineering and targeted mutagenesis have been emerged as attractive tool for strain improvement, though the classical mutagenesis methods (physical and chemical) in combination with mutant selection and medium optimization forms a prevailing strategy to obtain overproduction of the enzyme.

On an industrial scale, extracellular alkaline proteases are produced using complex media containing cost effective substrates responsible for 30 to 40% of the production costs (Kumar and Parrack, 2003). For potential industrial applications, organisms growing on economical substrates are requisite (Shikha et al., 2007). To this end, solid state (substrate) fermentation (SSF) offers utmost possibilities wherein agro-industrial wastes (wheat bran, rice bran, mustard oil cake, coconut oil cake etc.) are used for the production of value added products by using microorganisms. In addition, SSF poses several economic and engineering advantages over submerged fermentation (SmF) such as (a) low moisture content, preventing bacterial contamination, (b) simplicity of equipment (Hesseltine, 1987), (c) amenability to use upto 20-30% substrate, in contrast to the maximum of 5% in SmF process (Pamment et al., 1978) (d) high volumetric productivity (e) lower downstream processing charges (Pandey et al., 2000).

Keeping in view the incessant escalating demand of alkaline protease, the present study has been focused on the development of a potential mutant strain and optimization of

fermentation parameters in SSF to obtain elevated level of protease production.

EXPERIMENTAL: MATERIALS AND METHODS

Microorganism and culture conditions

A protease producing fungal strain was isolated in our laboratory from the soil sample of dairy industry and identified as *Aspergillus flavus* MTCC 9952 at Institute of Microbial Technology, Chandigarh, India. The culture was routinely maintained on malt extract-glucose-agar slants (containing g/L: malt extract, 20; glucose, 20; KCl, 0.5; MgSO4, 0.5; KH2PO4, 1.0; pH 9.0) at 4°C and was subcultured at every four weeks.

Strain improvement by chemical mutagenesis

The chemical mutagenesis was carried out by following the method of Punt et al. (1987). The spores from fully sporulated slants (7 day old) were harvested in sterile double distilled water containing Tween 80- 0.1% (v/v) and centrifuged at 10,000 xg for 10 min. The spores were washed twice with phosphate buffer (100 mM, pH 7.2) and spore concentration was set to 1x 10^7 spores/mL with the help of Neubauer haemocytometer. This spore suspension (200 µL) was mixed with 8 µL (200 µg/mL) of 4-nitroquinoline oxide (4-NQO, Sigma-Aldrich, USA) and 800 µL of sterile double distilled water. The mixture was incubated at 37°C for different time interval (10-90 min). After incubation, mutagen was inactivated by addition of 100 µL of 10% (w/v) sodium thiocynate followed by further incubation for 5 min. The mutagenized conidia were harvested by centrifugation at 10,000 xg for 5 min, washed with phosphate buffer (100 mM, pH 7.2) and resuspended in sterile distilled water.

Inoculum preparation

Prior to each experiment, the mutant strain was transferred to fresh slants and incubated at 37°C for 4 days. The spores from fully sporulated slant were dislodged and dispersed in 10 mL of 0.1% Tween 80 under aseptic conditions. Spore suspension (1 mL) was used as inoculum and viable spores in the suspension were determined (1x10^7 spores/mL) by serial dilution followed by plate count method.

Screening of potent mutant

Themutagenized spore suspension (0.2 mL) was spread on skimmed milk agar plates with composition g/L; skimmed milk, 50; $MgSO_4$, 0.5; KCl, 0.5; KH_2PO_4, 1.0, pH 9.0 and incubated at 37°C for 48 h.Thirty two mutants showing larger zone of hydrolysis around colonies were isolated from the plates exhibiting 70-99.9% lethality of spores.

Quantitative screening of all the mutants for improved alkaline protease producing property was done under SSF using basal medium. Wheat bran (5g) was dispensed in 250 mL Erlenmeyer flasks, moistened with 20 mL of Czapek dox solution (comprising g/L: $NaNO_3$, 25;KCl, 0.5; $MgSO_4$, 0.5; KH_2PO_4, 1.0; pH 9.0), sterilized, inoculated with 1 mL of spore suspension (1 x 10^7 spores) and incubated at 37°C for 4 days. Test flasks, in triplicate (a total of 12 flasks/mutant), were run separately for each day and respective flasks were harvested at every 24 h to determine the protease yield.

Optimization of process parameters for protease production by mutant strain

The optimization of fermentation parameters was performed in the basal medium (used for screening of mutants). Different physical and nutritional parameters affecting protease production were studied sequentially as under and the selected parameter was incorporated in the subsequent experiments: agro-industrial wastes, 5 g (wheat bran, WB; rice bran, RB; corn cob, CC; pigeon pea bran, PB; black gram bran, BgB; tea waste, TW) were tested alone and in combinations (ratio of 1:1) to find out the best substrate for protease production. The effect of incubation period (24 to 168 h) was evaluated followed by different ratios of WB and CC. The influence of initial moisture content on protease production was tested by varying the moisture content at different levels (44.4 to 87.5%). To study the effect of incubation temperature and initial pH, the fermentation medium was incubated at 32 to 45°C with initial pH adjusted to 8.5–10.5. Different inoculum levels [1x10^4 to 1x10^9 spores/gramofinitial dry substrate (IDS)] were also successively evaluated for optimum enzyme yield.

Different carbon sources (glucose, fructose, sorbitol, mannitol, galactose, sucrose lactose, starch soluble and maltose) were supplemented at the level of 100 mg/g IDS, in the fermentation medium. Similarly several organic (peptone, corn steep liquor, malt extract, yeast extract, casein and gelatin), inorganic (NH_2CONH_2, $(NH4)_2SO_4$, NH_4CL, KNO_3, NH_4NO_3, $(NH_4)_2HPO_4$ and $NH_4H_2PO_4$) nitrogen sources and metal ions ($CuSO_4$, $MnCl_2$, $ZnSO_4$, $CaCl_2$, $FeSO_4$ and $CoCl_2$) were also tested at the level of 50 mg/g IDS, 20 mg/g IDS and 0.5 mg/g IDS, respectively, in the

fermentation medium.

Enzyme extraction

Fermented bran (5 g) was soaked in 50 mL of sterile distilled water and agitated on a rotary shaker (120 rpm) for 2 h at 30°C. The resulted slurry was finally filtered and centrifuged at 10,000 xg for 10 min at 4°C. The ensued supernatant was recovered and used as crude enzyme extract.

Enzyme assay

Protease activity was determined as described by Hagihara et al. (1958). Suitably diluted supernatant (1 mL) was mixed with 1 mL of casein (1% w/v, prepared in 50 mM carbonate-bicarbonate buffer of pH 10.0) and incubated at 37°C for 10 min. The reaction was quenched by adding 3 mL of 10% pre-chilled trichloroacetic acid (TCA). The reaction tube was allowed to stand for 30 min for complete precipitation of proteins. The contents of the reaction tubes were filtered through Whatman No. 1 filter paper and absorbance of the filtrate was read at 275 nm which was extrapolated against tyrosine standard curve. A unit of protease activity is defined as the amount of enzyme liberating 1µg tyrosine/mL/min under the assay conditions.The protease activity was reported as per gram of solid substrate used for protease production.

Statistical analysis

All the experiments were carried out in triplicates and the results are presented as the mean of three independent observations. The standard deviation for each set of experimental result was calculated using Microsoft Excel.

RESULTS AND DISCUSSION

Strain improvement by chemical mutagenesis

Strain improvement through mutation and screening of a potent mutant could be the key for the successful development of industrially viable strains. The wild strain NSD 08 was subjected to chemical mutagenesis and an improved mutant strain (MNSD 29) was isolated which produced 24% higher alkaline protease than wild strain. However, no significant alteration was observed in the general morphology of mutant strain. In earlier studies Djamel et al. (2009) developed a mutant strain of Penicillium sp producing 42% higher acidic protease and Shikha et al. (2007) reported 44% enhanced alkaline protease production by a mutant of Bacillus pantotheneticus.

Effect of type of solid substrate and incubation period on protease production

The extent of enzyme production was monitored as a function of type of substrate alongwith incubation period. Potential of agro-industrial wastes as substrate for alkaline protease production was studied and observations are presented in Table I which suggested that the most suitable substrate for this mutant strain was mixture of WB and CC in the ratio of 1:1 with respect to enzyme yield (1121 U/g IDS) and incubation period (48 h). However, other substrates like PB, BgB and WB (alone) also tend to support sufficient enzyme (1057, 1075 and 1011 U/g IDS, respectively) production but required 72, 72 and 96 h of incubation, respectively.

Various substrates (with varying composition) are preferred by different microbial strains for their growth and enzyme production which may be governed by their metabolic properties. Several agro by-products were screened by various workers for protease production, Agrawal et al. (2005) and Sandhya et al. (2005) foundwheat bran as most effective in terms of protease production after 168 h and 72 h of incubation. However, Sumantha et al. (2006) reported rice bran and Nehra et al. (2002) reported mixture of rice bran, rice husk and gram hull in the ratio 5:3:2 to be suitable for protease production.

Table 1

Effect of type of substrate along with incubation period for protease production

Type of substrate	Enzyme activity (U/g IDS)					
	48 h	72 h	96 h	120 h	144 h	168 h
TW	229±24	309±31	473±21	525±29	499±33	350±23
WB	336±19	578±25	1011±56	775±31	565±27	477±36
RB	343±31	486±24	621±45	518±22	287±51	301±21
PB	648±26	1057±54	721±49	508±36	496±66	289±47
BgB	718±25	1075±53	713±26	646±47	686±41	422±26
WB+RB	331±12	499±54	652±37	476±40	449±28	236±33
PB+WB	516±34	767±51	751±28	507±50	377±24	348±28
WB+CC	1121±74	847±67	765±49	719±62	583±35	463±47
BgB+WB	449±48	829±72	1022±68	751±32	687±56	524±42
BgB+CC	405±58	822±51	653±78	578±39	490±43	309±45

TW- tea waste, WB- wheat bran, RB- rice bran, PB- pea bran, BgB- black gram bran, CC- corn cob

Different substrates (5g), dispensed in 250 mL Erlenmeyer flasks, moistened with 20 mL of Czapek dox solution (pH 9.0), inoculated with 1 mL (1x107 spores/ mL) of spore suspension and incubated at 37°C for 24 to 168 h. ± Values reflects the mean standard deviation of triplicates of three independent experiments.

Effect of initial moisture content on protease production

Table II showed the effect of initial moisture content on protease production by mutant strain. An initial moisture content of 50% was found to be optimum (1895 U/g IDS). The protease production decreased on both the sides of this optimum level. Identical results were reported by Chutmanop et al. (2008) and Agrawal et al. (2004) for protease production by Aspergillus oryzae and Penicillium sp., respectively. However, Sandhya et al. (2005) and Tunga et al. (1998) reported 43.6 and 140% initial moisture content as optimum for A. oryzae and Rhyzopus oryzae, respectively.

According to Zandrazil and Brunert (1981) in SSF lower moisture levels lead to reduced solubility of the nutrients in the solid substrates, a lower degree of substrate swelling and higher water tension. Similarly, higher moisture contents cause decreased porosity, loss of particle structure, clumping of substrate particles, reduction in gas volume, decreased gas exchange and enhanced formation of aerial mycelium (Lekha and Lonsane, 1994) resulted in decrease in protease yield.

Table 2

Effect of initial moisture content on protease production

Initial moisture content (%)	Enzyme activity (U/ g IDS)
44.4	1340 ± 72
50.0	1895 ± 86
66.7	1625 ± 105
75.0	1351 ± 51
80.0	1047± 59
83.3	1057 ± 62
85.7	858 ± 54
87.5	580 ± 63

Wheat bran and corn cob (5g, in ratio of 1:1) moistened with different volumes of Czapek dox solution (pH 9.0) to produce desired initial moisture content. Inoculated flasks were incubated at 37°C for 48 h. ± Values reflects the mean standard deviation of triplicates of three independent experiments.

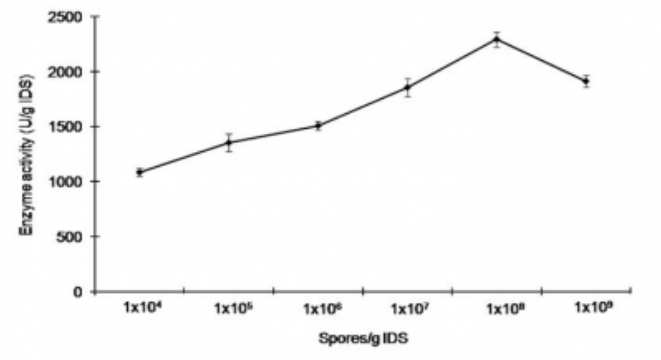
Effect of inoculum size on protease production

There was gradual increase in the enzyme yield with increasing inoculum size and maximum enzyme (2253 U/g IDS) was observed at the level of 1x108 spores/g IDS (Fig. 1). Further augmentation of inoculum resulted in decline in protease production. The nature of inoculum as well as its size may affect the microbial process (Elibol et al., 1995) and determines the biomass production in fermentation (Sandhya et al., 2005). The diminution in enzyme yield with larger inoculum size (> 1x108) could be attributed to the shortage of nutrients available for the larger biomass and faster growth of the culture (Hesseltine et al., 1976). However, lower inoculum sizes lead to the under

development of biomass resulted into less protease production since the protease production was growth associated (data not shown).

Figure 1

Effect of inoculum size on protease production.



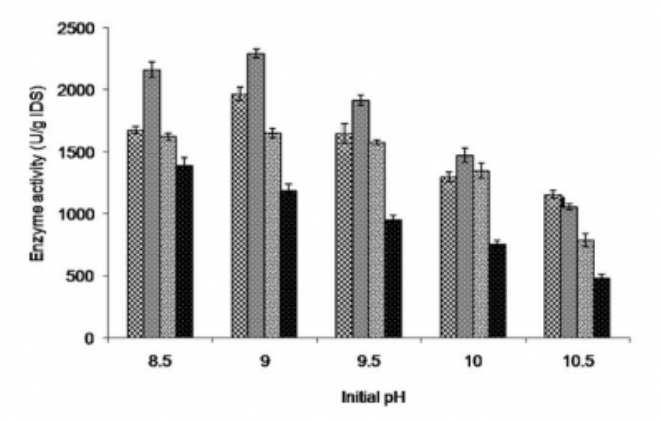
Fermentation medium with initial moisture content 50%, initial pH 9.0, inoculated with different levels of inoculums sizes (1x104 to 1x109 spores/g IDS) were incubated at 37°C for 48 h. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments.

Effect of incubation temperature and initial pH on protease production

The impact of pH and temperature on protease production is presented in Fig. 2. The incubation temperature has a profound effect on the enzyme yield and duration of enzyme synthesis phase (Ramesh and Lonsane, 1987). Likewise, the pH of the medium regulates many enzymatic processes and transport of various components across the cell membrane (Moon and Parulekar, 1991). It is clear from the observations (Fig. 2) that optimum temperature and pH for mutant were 37°C and 9.0, respectively, for protease production (2291 U/g IDS). However, mutant strain was able to grow in all ranges of pH and temperature except pH 10.5 and 45°C where limited growth and as a consequence less protease production was observed. Furthermore, decreased level of protease at higher temperature may be due to protease autolysis and thermal inactivation at the fermentation temperature (Murphy and Fagain, 1996). Several reports (Sindhu et al., 2009; Vishwanatha et al., 2010) suggested protease production in the range of 30-35°C with mesophilic organisms. Correspondingly, Anandan et al. (2007) and Hajji et al. (2007) reported alkaline protease production at pH 9.0 and 8.0 with A. tamari and A. clavatus, respectively.

Figure 2

Effect of temperature along with initial pH on protease production.



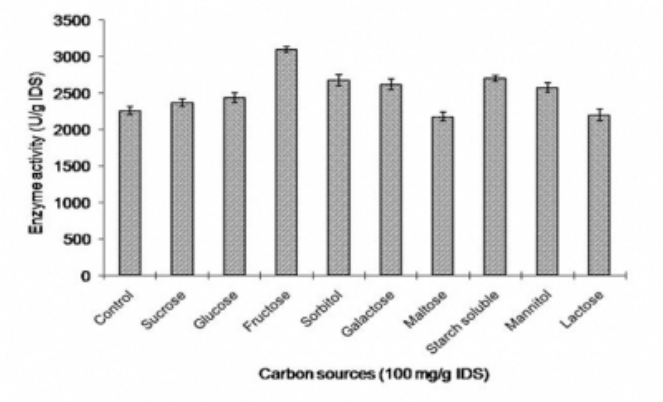
Fermentation medium with initial moisture content 50%, initial pH 8.5 to 10.5, inoculated with 1x108 spores/g IDS were incubated at temperatures 32 (first bar), 37 (second bar), 40 (third bar) and 45°C (fourth bar) for 48 h. triplicates of three independent experiments.

Effect of carbon supplementation on protease production

An attempt was made to study the effect of addition of carbon sources on protease production (Fig. 3). Observations revealed that fructose favoured the maximum enzyme production (3098 U/g IDS) followed by soluble starch (2700 U/g IDS) and sorbitol (2677 U/g IDS). All the other carbon sources exerted marginal positive effect on protease production except maltose and lactose. Suitability of carbon source vary with the organisms as Tremacoldi and Carmona (2005) reported glucose for A. clavatus and Abidi et al. (2008) found starch for Botrytis cinerea, respectively, as best carbon source. To determine the optimum level of the fructose for enzyme production different concentrations (50 – 250 mg/g IDS) were also used in the medium and maximum protease (3419 U/g IDS) was obtained at a concentration of 200 mg/g IDS (data not shown). Further increase in the fructose concentration resulted drop in protease production.

Figure 3

Effect of carbon supplementation on protease production.



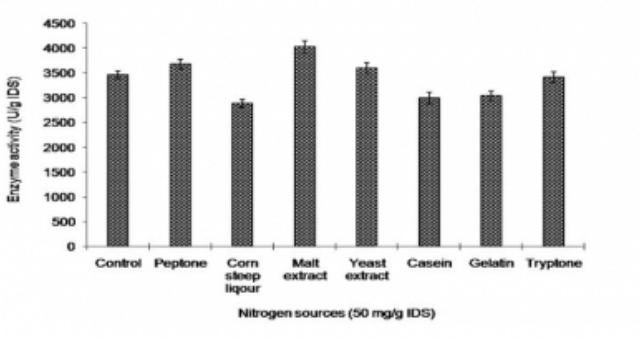
Inoculated fermentation media with initial moisture content 50%, initial pH 9.0, supplemented with different sugars (100 mg/g IDS) were incubated at 37°C for 48 h. The control represents absence of any sugar. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments.

Effect of protein augmentation on protease production

Severalorganic nitrogen sources were investigated to monitor their effect on protease production (Fig. 4). Although peptone (3670 U/g IDS) and yeast extract (3591 U/g IDS) supported enhanced level of enzyme production but malt extract showed maximum enzyme yield (4022 U/g IDS). The addition of casein (2989 U/g IDS), gelatin (3031 U/g IDS) and corn steep liquor (2879 U/g IDS) negatively affected the protease production, whereas, other sources slightly increased the protease yield. Chellapan et al. (2006) also reported the malt extract as best organic nitrogen source for protease production by Engyodontium album. In contrast, Chakraborty et al. (1995) reported casein as best nitrogen source for A. niger. Further increase in the concentration of malt extract increased the protease production and maximum yield (4381 U/g IDS) was obtained at concentration of 100 mg/g IDS (data not shown).

Figure 4

Effect of protein augmentation on protease production.



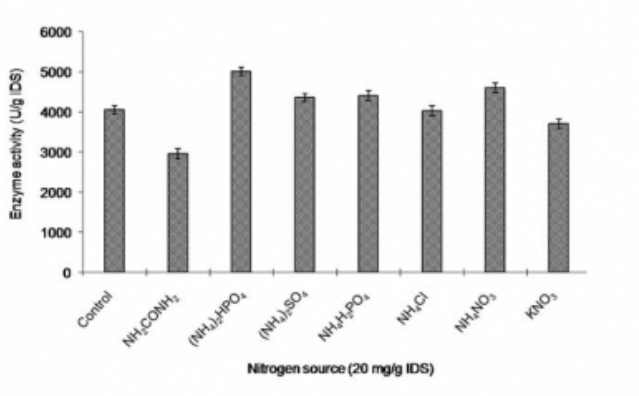
Inoculated fermentation media with initial moisture content 50%, initial pH 9.0, supplemented with different organic nitrogen sources (50 mg/g IDS) were incubated at 37°C for 48 h. The control represents absence of any nitrogen source. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments.

Effect of inorganic nitrogen sources on protease production

Of the inorganic nitrogen sources tested, di-ammonium hydrogen phosphate (5009 U/g IDS) had a profound effect on protease production (Fig. 5). Similar results were also reported by Srinubabu et al. (2007) for A. oryzae. Extracellular protease was not detected when the organism was grown in the presence of urea or potassium nitrate. Chellappan et al. (2006)alsofound that urea completely inhibited protease production. However, other nitrogen sources showed insignificant increase on protease production.

Figure 5

Effect of inorganic nitrogen on protease production.



Inoculated fermentation media with initial moisture content 50%, initial pH 9.0, supplemented with different inorganic nitrogen sources (20 mg/g IDS) were incubated at 37°C for

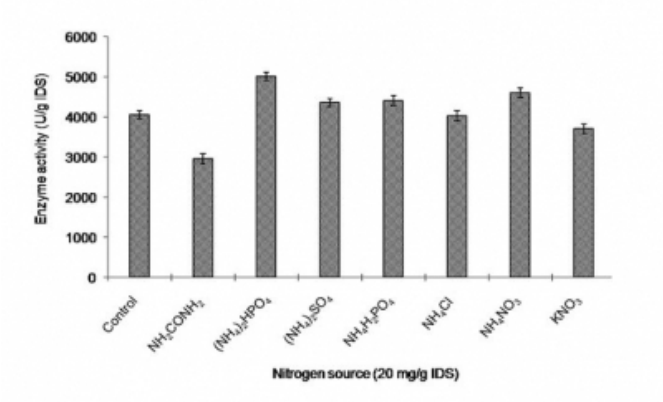
48 h. The control represents absence of any nitrogen source. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments

Effect of metal salts on protease production by mutant strain

Fig. 6 depicts the effect of metal salts on protease production. According to Varela et al. (1996), metal ions in the fermentation medium greatly influence the protease production by microbes. Observations revealed that CuSO4 supported the maximum enzyme production (5653 U/g IDS), followed by CaCl2 (5388 U/g IDS), whereas, MnCl2, ZnSO4 and CoCl2 inhibited the enzyme production.

Figure 5

Effect of inorganic nitrogen on protease production.



Different metalions (0.5 mg/g IDS) were supplemented in the optimized medium and incubated at 37°C for 48 h. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments.

CONCLUSION

The overproducing strains are always the choice of organism to fulfill the current exponentially increasing demand of proteases due to applications in several industries. Chemical mutagenesis followed by medium optimization increased protease yield by 1.37 fold after 48 h of incubation which was comparable to bacteria. Agro by-products, abundant in occurrence in country like India, could fruitfully be used as nutrients for production of proteases at industrial level using filamentous fungi under solid state fermentation. The agro wastes itself act as complex carbon and nitrogen sources but addition of carbon and nitrogen sources further augment the enzyme production at optimum level by facilitating early and robust biomass development.

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References

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