Advanced Journal of Microbiology Research Volume 2014
Available online at http://advancedresearchjournals.org/AJMR

Full Length Research Paper

Optimization of process parameters for the production of alkaline protease from *Penicillium godlewskii* SBSS 25 and its application in detergent industry

R. Sindhu*, G. N. Suprabha and S. Shashidhar

Physiological Chemistry Research Laboratory, School of Biosciences, Mahatma Gandhi University, Kottayam-686 560, Kerala, India.

Accepted 23 July, 2013

An investigation was carried out on alkaline protease production using solid state fermentation from *Penicillium godlewskii* SBSS 25 isolated from soil samples collected from Southern Kerala, India and its application as a detergent additive. The maximum protease yield (235 U/gds) were achieved with optimized process parameters like pH (9.0), temperature (35°C), moisture content (60%), nitrogen source (0.5% NH_4NO_3), carbon source (4% glucose) and incubation time (96 h). The enzyme showed stability with a wide range of commercial detergents. These properties make this protease an ideal choice for application in detergent formulations.

Key words: Extracellular, alkaline protease, *Penicillium godlewskii*, optimization, soil, fermentation.

INTRODUCTION

Protease constitutes a large and complex group of enzyme which plays an important nutritional and regulatory role in nature. A variety of microorganisms such as bacteria, fungi, yeasts and actinomycetes are known to produce this enzyme (Reese et al., 1950; Taguchi et al., 1983; Kim et al., 1993; Manjeet et al., 1998). Commercially proteases are the most important industrial enzymes accounting for about 60% of the total enzyme market (Ng and Kenealy, 1986). The use of proteases as detergent additives in the1960s was responsible for their large scale commercial development and also for a considerable expansion in fundamental research in these enzymes. The present investigation deals with the isolation of proteolytic fungi from soil samples collected from various parts of Kerala and their *in vitro* production of extracellular protease, which have potential applications in detergent industry.

MATERIALS AND METHODS

Isolation of fungal strains

Penicillium godlewskii SBSS 25 used in the present investigation was isolated from soil samples collected from various parts of Southern Kerala, India. Fungal isolation was done by serial dilution plating on Sabourauds dextrose agar media. Protease producing strains were selected by spotting the fungal cultures on casein agar media and incubated at room temperature for 48 h (Ergin and Semra, 1994). The strains which produce protease exhibit zone of clearance around the colony.

Production media

Wheat bran procured from local market was used as solid substrate. Solid state fermentation was carried out in 250 ml Erlenmeyer flask containing 20 g of wheat bran moistened to 60% with salt solution containing (g/l) $NaNO_3$ - 3, KH_2PO_4 - 1, KCl - 0.5, $FeSO_4.7H_2O$ - 0.01, Glucose - 30, pH-9 (Ellaiah et al., 2002).

Culture conditions

Spores of the selected fungus were harvested from seven day old slant cultures by suspension in sterile distilled water containing 0.01% Tween-80 (Ramachandran et al., 2004; Agrawal et al., 2005). The spore suspension diluted to desired count (5×10^7 spores /ml) served as an inoculum. Inoculum was added to 20 g wheat bran in a 250 ml conical flask and was moistened to 60% water content with salt solution. The flasks were incubated at 35°C for 96 h under stationary conditions in a BOD incubator. After incubation, protease was extracted by shaking for 30 min with 50 ml distilled water. Solids were separated by squeezing through two fold cheese cloth and then filtering through Whatman No. 1 filter paper. The filtrate was used as source of protease.

*Corresponding author. E-mail: sindhufax@yahoo.co.in. Tel: +91-474-2740696, +91-9947341947.

Protease assay

The protease activity was assayed by casein digestion method (Kunitz, 1947). The reaction mixture contained suitably diluted enzyme and casein in 0.1M sodium carbonate buffer pH 10. The reaction mixture was incubated at 40°C for 10 min. The reaction was terminated by the addition of 3 ml of 10% trichloroacetic acid. The terminated reaction mixture was incubated at room temperature for 30 min. The precipitate formed was filtered through Whatman No. 1 filter paper. The absorbance of the filtrate was measured at 280 nm. Tyrosine was used as standard. One unit of protease activity is defined as the amount of enzyme which liberates one micromoles of tyrosine per minute per gram dry substrate under experimental conditions. Protein was estimated by the method of Lowry et al. (1951).

Optimization of process parameters for enzyme production

Various process parameters influencing enzyme production during SSF were optimized. The strategy followed was to optimize each parameter, independent of the others and subsequently optimal conditions were employed in all experiments (Uyar and Bysal, 2004).

The effect of process parameters on enzyme production was determined by incubating at different pH (4 - 10), temperature (30 - 55°C), moisture content (20 - 80%), carbon source, nitrogen source, incubation time (24 - 120 h) and glucose concentration (2 - 10%).

Effect of initial pH: Initial pH plays an important role in SSF. For optimization of initial pH on enzyme production, the pH of the salt solution was adjusted in the range of 4 - 10 with 1N HCl or 1N NaOH. The other conditions were moisture content of 60% and inoculums concentration of 5 x 10^4 spores and the fermentation was carried out for 96 h at 30°C.

Effect of temperature: 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 30 - 55°C keeping other conditions constant.

Effect of moisture content: Initial moisture content plays an important role in SSF. To study the effect of initial moisture content on enzyme production, the SSF was carried under different moisture content (20 - 80%) of wheat bran by varying the amount of salt solution used to moisten the wheat bran. The optimum moisture content achieved by this step was used for subsequent experiments.

Effect of carbon source: To study the effect of carbon source on enzyme production, SSF was carried out using various simple and complex carbon source like sucrose, glucose, starch, mannitol, lactose and fructose (3.0% w/v) keeping other conditions constant.

Effect of nitrogen source: To study the effect of nitrogen source on enzyme production, SSF was carried out using various nitrogenous salts such as NH_4NO_3, KNO_3, $NaNO_3$, NH_4Cl, $(NH_4)_2CO_3$, $NaNO_2$, $(NH_4)_2SO_4$ incorporated at 0.5%(w/v) in the medium keeping the other conditions constant.

Effect of incubation time: The effect of incubation time on enzyme production by the fungi under SSF was studied by incubating the inoculated flask for a total period of 120 h and estimating the enzyme production at regular intervals of 24 h.

Effect of glucose concentration: To study the effect of glucose concentration on enzyme production, SSF was carried out with different glucose concentration (2 - 10% w/v) keeping other conditions constant.

Stability of enzyme in presence of commercially available detergents

Different commercially available detergents like Surf, Henko, Ariel and Tide were used to study the compatibility of the alkaline protease. The enzyme was incubated in presence of these detergents in a concentration of 7 mg/ml and incubated at 30°C for 24 h. Enzyme activity without any detergent was taken as 100%. Residual activity was determined and the relative activity was determined (Joo and Chang, 2006).

RESULTS AND DISCUSSION

The culture of *P. godlewskii* SBSS 25 effectively colonized the surfaces of wheat bran in solid state fermentation and exhibited dense growth. Wheat bran produces maximum protease concentrations after four days of incubation. The particle size and chemical composition of the substrate are of critical importance (Hesseltine, 1987). The suitability of wheat bran is apparent from the sufficient nutrient that it contains and its ability to remain loose even in the moist state where it provides large surface area and aerobic conditions.

Effect of pH

The pH of the medium was adjusted to different levels (4 - 10). *P. godlewskii* SBSS 25 showed maximal protease production at pH - 8, although significant levels of protease could be recorded at other pH (Figure 1). This indicated the alkalophilic nature of the isolate. Identical observation was reported in *Bacillus* species (Patel et al., 2005). The strain was able to grow in the pH range 5.0 to 10.0, with better protease yield in the alkaline range. The pH below 5.0 and above 9.0 was not conducive for mycelial growth and had the adverse effect on enzyme production. Contrary observations were earlier recorded in *Aspergillus flavus, Aspergillus oryzae* and *Aspergillus candidus* at pH 4.0 (Nasuno and Onara, 1972; Dworschack et al., 1952).

Effect of temperature

The medium was incubated at various temperatures (30 - 55°C). *P. godlewskii* SBSS 25 showed maximal protease production at 35°C (Figure 2). Significant range of protease was produced at temperature ranging from 30 - 45°C.

Medium temperature plays an important role in the protease production (Sinha and Satyanarayana, 1991). Optimum temperature for the production of protease by *P. godlewskii* SBSS 25 was 35°C. Identical observations were earlier recorded in *A. flavus, A. oryzae* and *A. candidus* (Nasuno and Onara, 1972; Dworschack et al., 1952). *P. godlewskii* SBSS 25 was able to grow in a temperature range of 30 - 45°C with better protease production at 35°C. Temperature below 35°C as well as above 45°C was not conducive for mycelial growth and

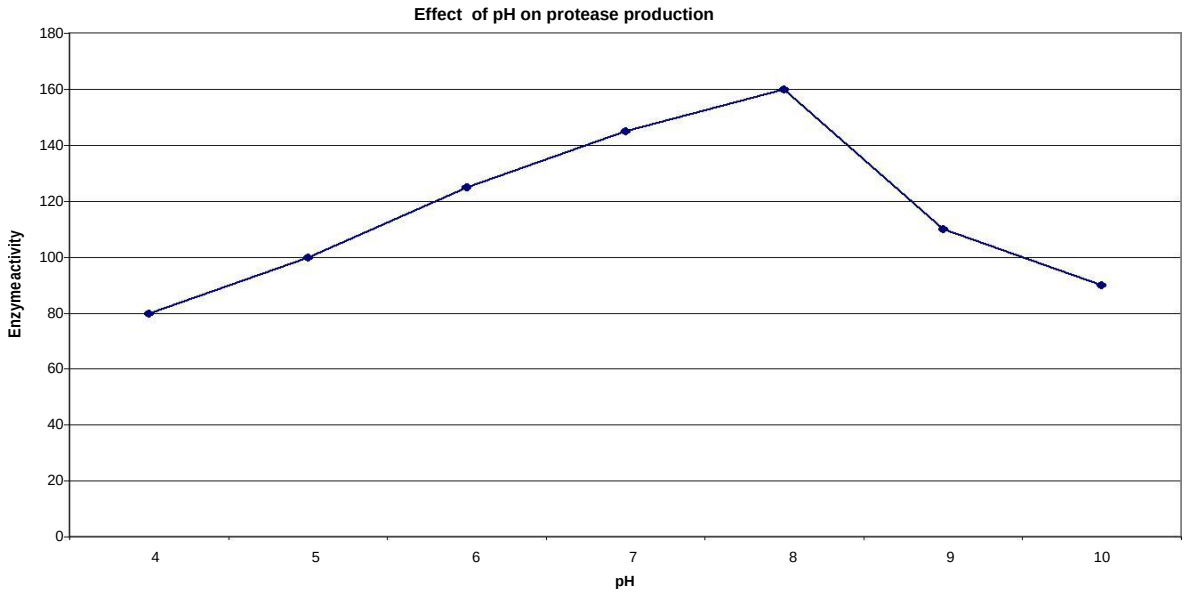

Figure 1. Effect of pH on protease production by *Penicillium godlewskii* SBSS 25.

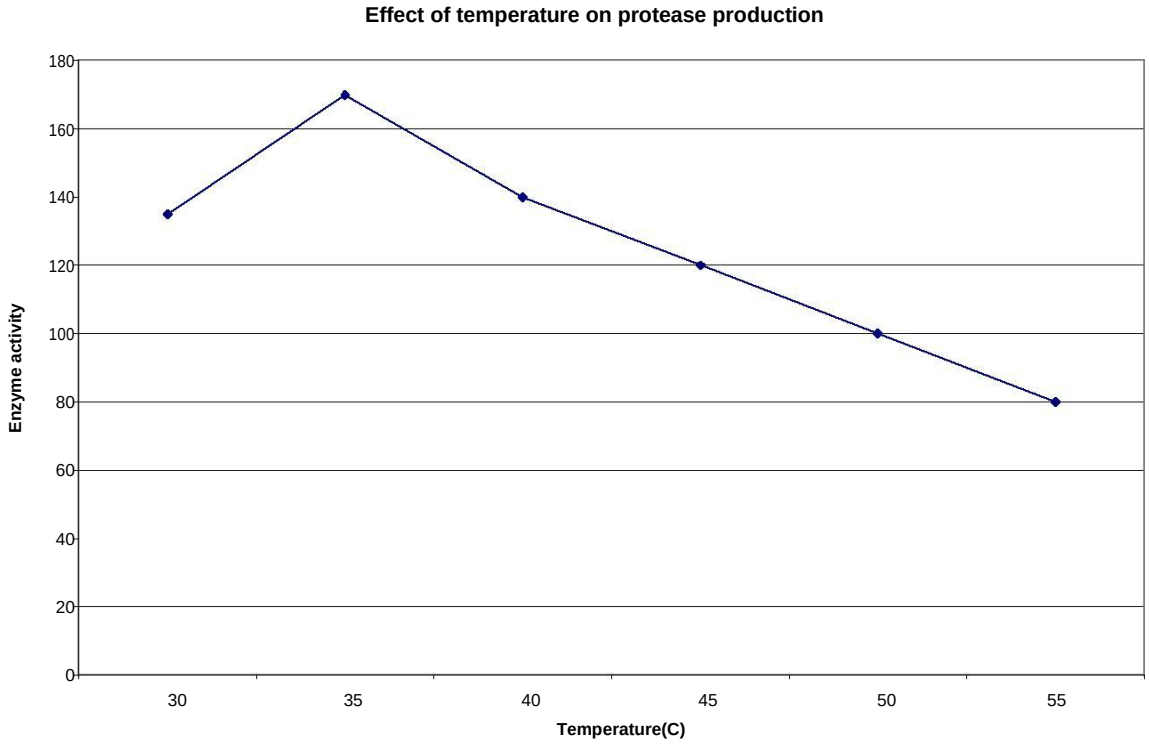

Figure 2. Effect of temperature on protease production by *Penicillium godlewskii* SBSS 25 on SSF.

has the adverse effect on enzyme production.

Effect of moisture content

Moisture content plays an important role in protease production (Aidoo et al., 1982). Optimum moisture content for the production of protease by *P. godlewskii* SBSS 25 was 60% (Figure 3). Identical observations were earlier recorded in *Rhizopus oligosporus* and *Bacillus amyloliquefaciens* (Pandey et al., 1999). *P. godlewskii* SBSS 25 *was* able to grow in the range of 30 - 60% moisture content with better protease production at 60% moisture

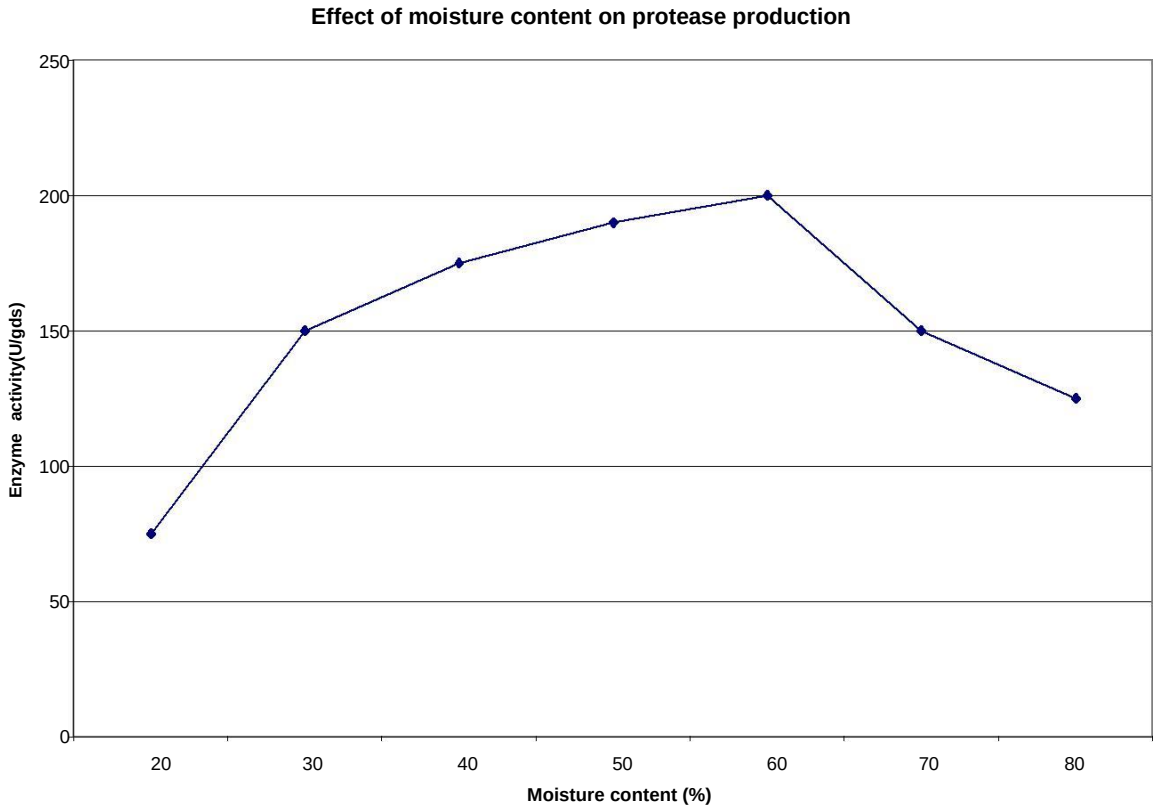

Figure 3. Effect of moisture content on protease production by *Penicillium godlewskii* SBSS 25 *on* SSF.

content. High moisture content was not conducive for mycelial growth due to agglomeration of wheat particles which in turn reduce oxygen level.

Effect of nitrogenous salts

Nitrogenous salts such as NH_4NO_3, KNO_3, $NaNO_3$, NH_4Cl, $(NH_4)_2CO_3$, $NaNO_2$, $(NH_4)_2SO_4$ were incorporated at 0.5% level in the medium. *P. godlewskii* SBSS 25 showed highest activity in presence of NH_4NO_3 followed by KNO_3 and $NaNO_3$. It showed lowest activity in the presence of $(NH_4)_2CO3$ (Figure 4).

Certain nitrogenous salts tend to decrease the pH of the culture medium and had the adverse effect on enzyme production, although they supported the growth of the organism (Wang et al., 1974).

Effect of carbon source

Carbon source such as glucose, fructose, lactose, sucrose, starch and mannitol were incorporated at 3% level in the medium. Sucrose and lactose did not accelerate protease production. The best carbon source for protease production by *P. godlewskii* SBSS 25 was glucose, while mannitol and starch was comparable (Figure 5). Accelerated protease production by glucose was earlier reported for *Conidiobolus coronatus* (Laxman et al., 2005).

Mannitol stimulated protease synthesis as they do not affect the pH of the culture medium or growth of the organism (Sen and Satyanarayana, 1993). Sugars appear to suppress protease release in fungi and other microorganisms (Drucker, 1972).

Effect of incubation time

The solid state fermentation medium was inoculated with the fungal strain and incubated for various time intervals (24 -120 h).

Figure 6 shows the effect of incubation time on protease production. *P. godlewskii* SBSS 25 produced highest amount of enzyme on the fourth day of incubation. Gradual increase in enzyme production was observed reaching its maximum on fifth day. *A. flavus, A. oryzae* and *A. candidus* showed maximum enzyme production on fourth day (Klapper et al., 1973; Ikushima et al., 1989).

Effect of glucose

When glucose was compared for protease production with using different concentration of glucose (2 to 10%) it maximal enzyme production, although significant level of protease was produced at glucose concentration ranging

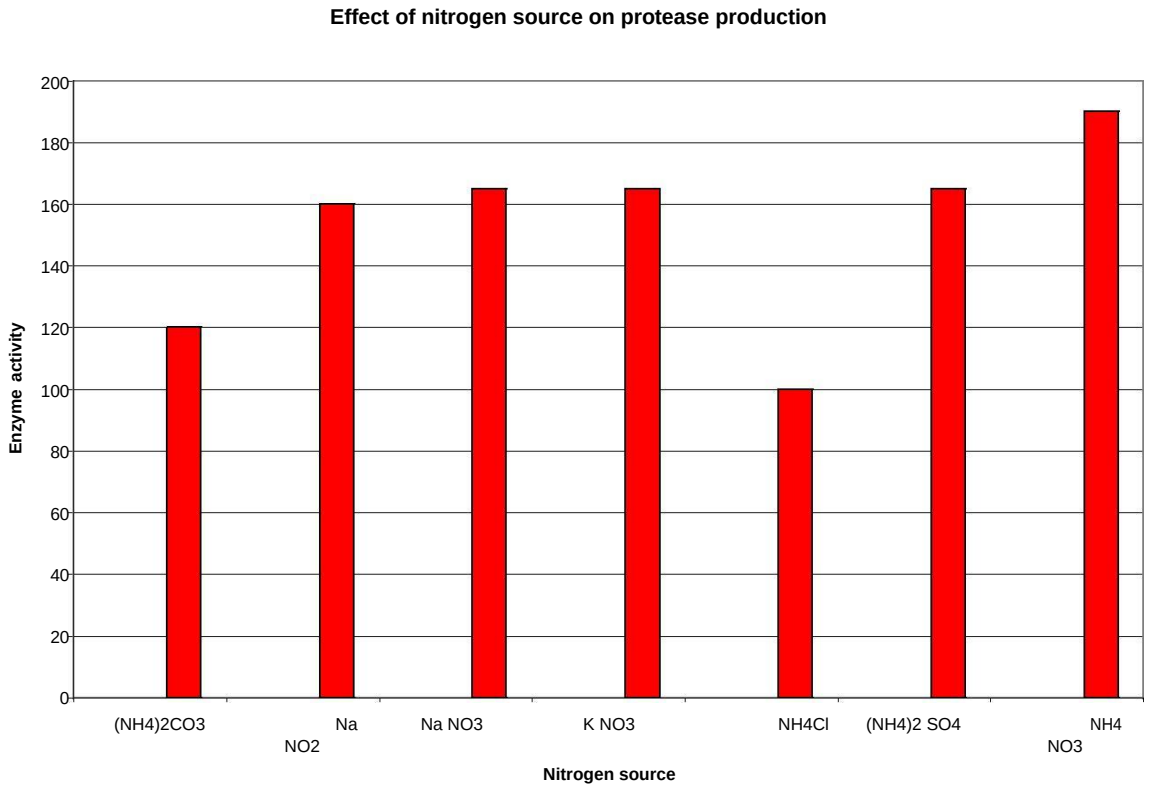

Figure 4. Effect of inorganic nitrogen source on protease production *Penicillium godlewskii* SBSS 25 on SSF.

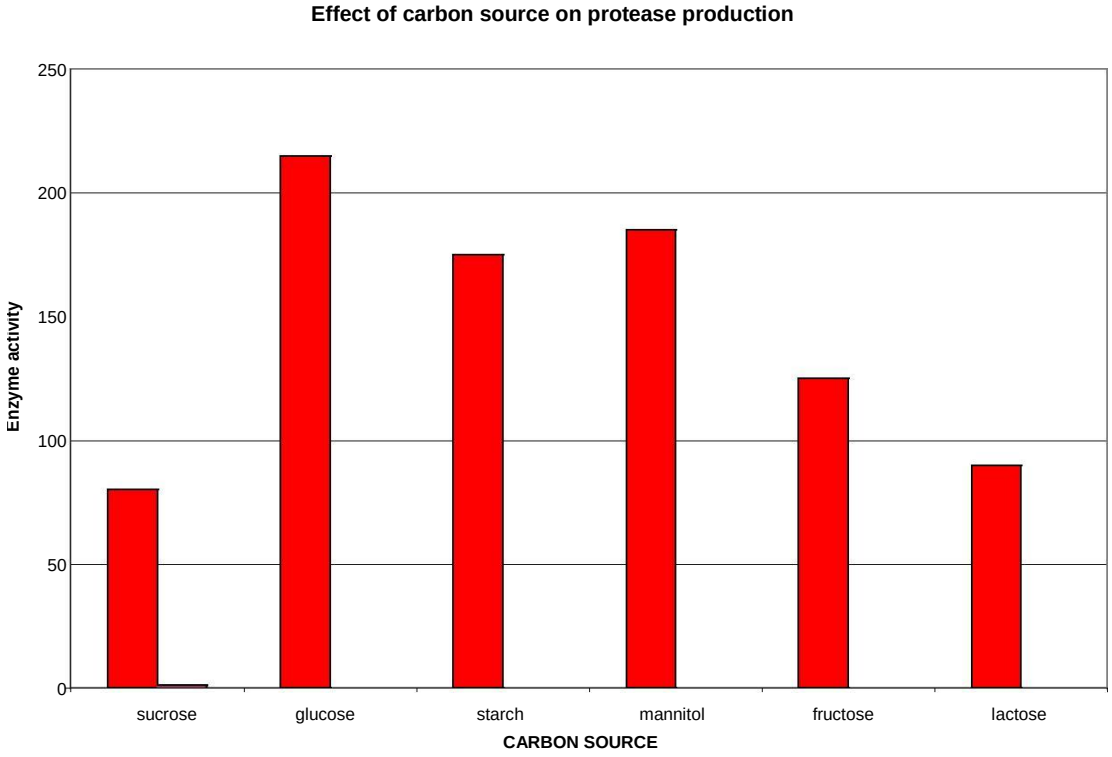

Figure 5. Effect of additional carbon source on protease production by *Penicillium godlewskii* SBSS 25 on SSF.

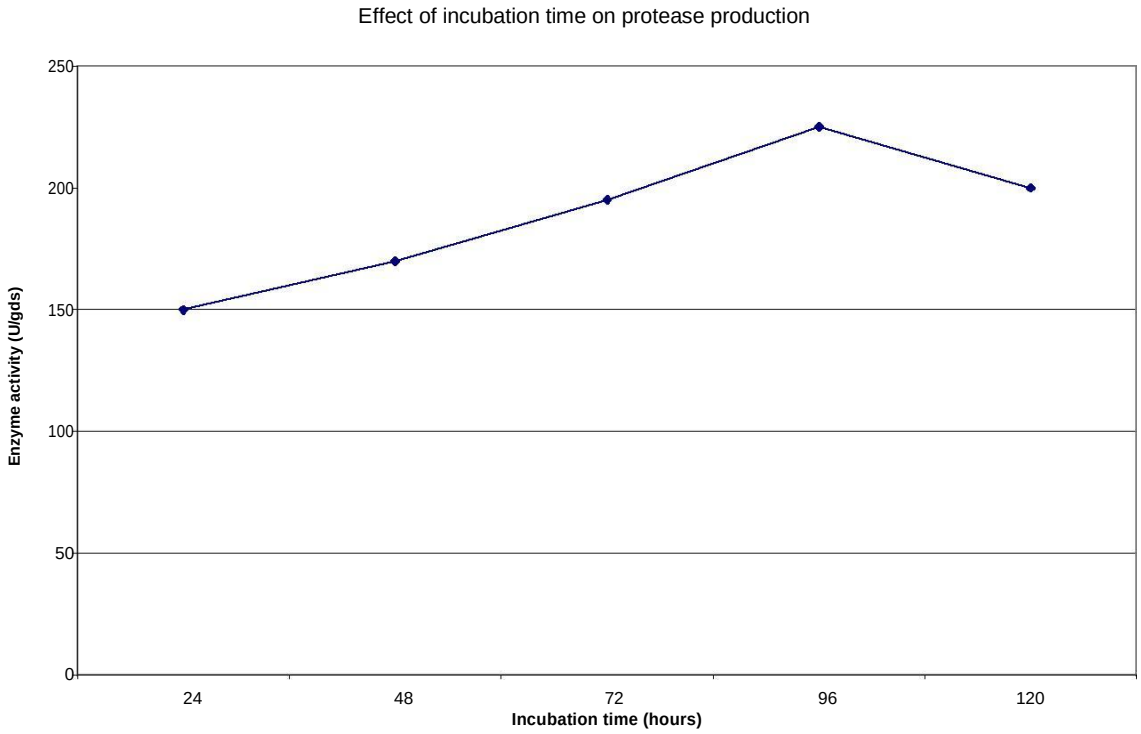

Figure 6. Effect of incubation time on protease production by *Penicillium godlewskii* SBSS 25 on SSF.

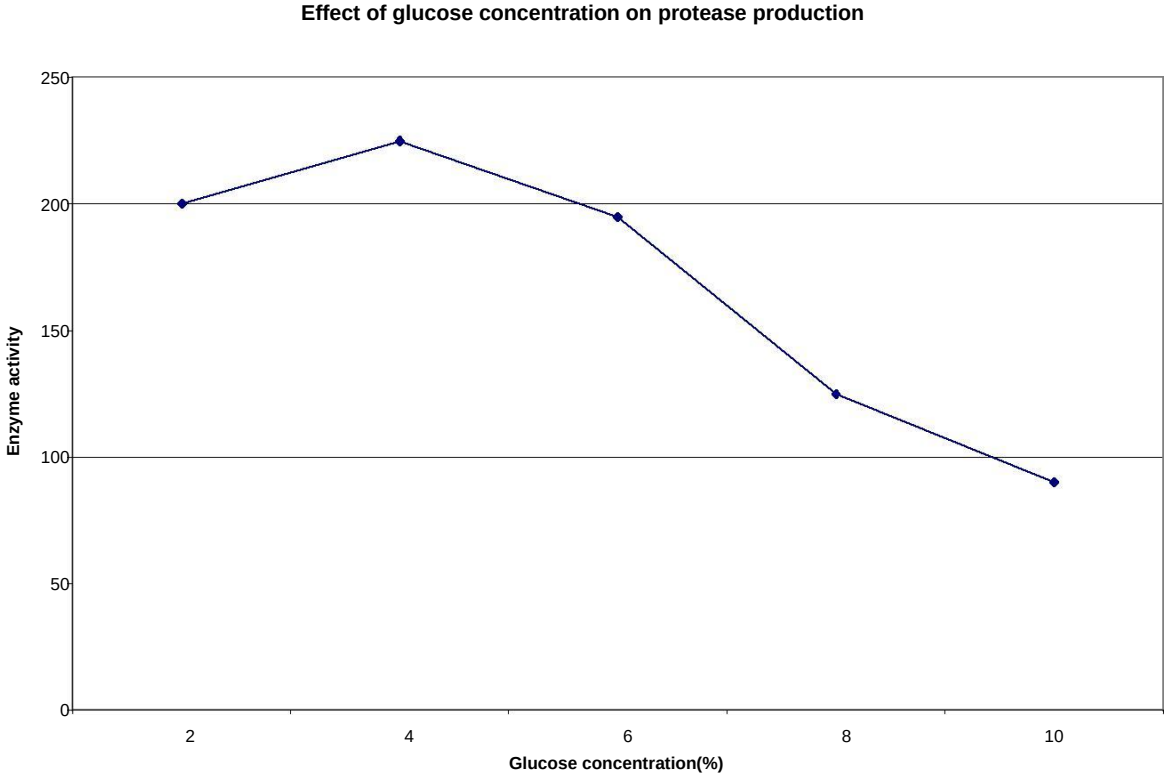

Figure 7. Effect of glucose on protease production by *Penicillium godlewskii* SBSS 25 on SSF.

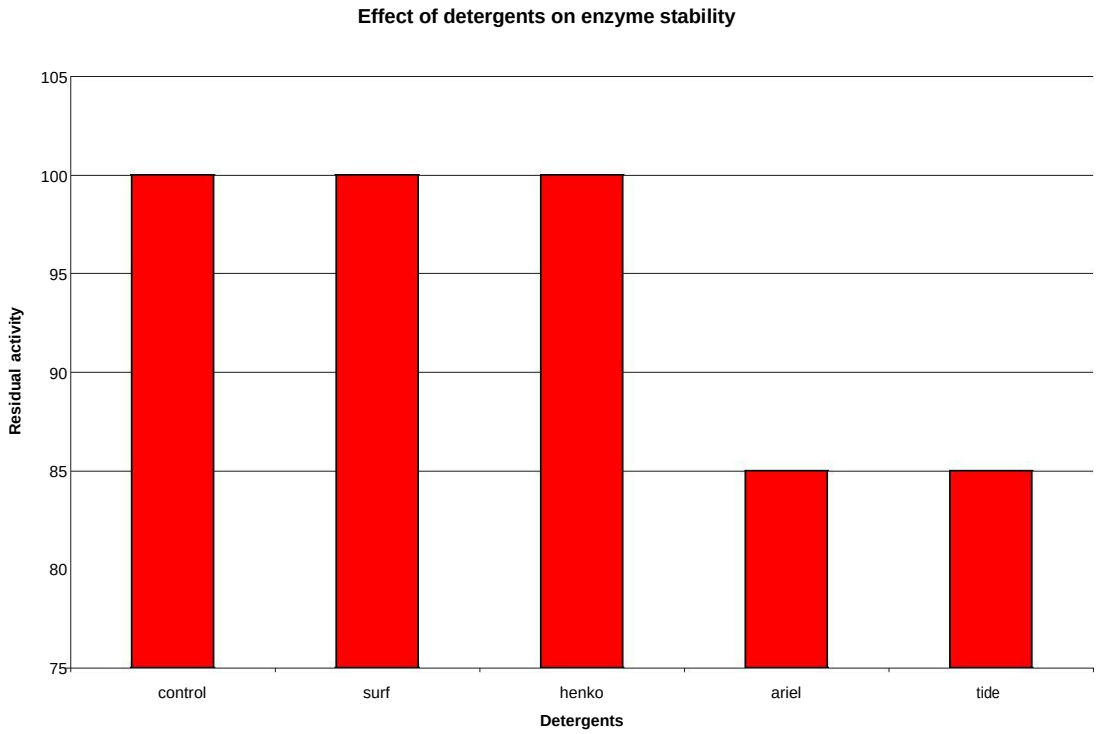

Figure 8. Effect of detergents on stability of protease from *Penicillium godlewskii* SBSS 25.

from 2 to 4% compared to other concentration (Figure 7). Increase in concentration of glucose resulted in lesser secretion of protease.

Stability of enzyme in presence of commercially available detergents

The enzyme was found to be 100% stable in presence of commercially available detergents like Surf and Henko and 85% stable in presence of Ariel and Tide (Figure 8). Stability of protease for laundry detergents was earlier reported by Venugopal and Saramma (2006) for alkaline protease produced by *Vibrio fluvialis* strain VM10 isolated from mangrove sediment sample.

P. godlewskii SBSS 25 was reported for the first time to produce alkaline protease which finds application in detergent industry. Based on the results reported here, it is proposed that the alkaline protease produced by *P. godlewskii* SBSS 25 isolated by us may have great commercial value as sufficiently high levels of enzyme activity compared to other *Penicillium* species reported and also high stability in the presence of detergents. The appreciably high enzyme activity makes *Penicillium* species an industrially promising and of special interests for basic and applied research.

REFERENCES

Agrawal D, Patidar P, Banerjee T, Patil S (2005). Alkaline protease production by a soil isolate of *Beauveria feline* under SSF condition: Parameter optimization and application to soy protein hydrolysis. Process Biochem. 40: 1131–1136.

Aidoo EK, Henry R, Wood BJB (1982). Microbial Proteases. Adv. Appl. Microbiol. 28: 201- 237.

Drucker H (1972). Regulation of extracellular protease in *Neurospora crassa*- Induction and repression of enzyme synthesis. J. Bacteriol. 110: 1041-1049.

Dworschack RG, Koepsell HJ, Lozada AA (1952). Evaluation of proteases produced by molds of the *Aspergillus flavus – oryzae* group in submerged culture. Biochem. Biophy. 41: 48- 60.

Ergin, Semra K (1994). Detection of proteinase activity in *Candida albicans* strains isolated from clinical samples with casein-agar method. Microbiol. Bull. 28: 338 - 344.

Ellaiah P, Adinarayana K, Bhavani Y, Padmaja P, Srinivasulu B (2002). Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergillus* species. Process Biochem. 38: 615- 620.

Hesseltine CW (1987). Solid state fermentation – An overview. Int. Biodeterior. 23: 79 -89.

Ikushima Y, Itoh H, Fukase T, Motai H (1989). Continuous protease production in a chemostat culture by salt tolerant *Aspergillus oryzae*. Appl. Microbiol. Biotechnol. 30: 604 – 608.

Joo H, Chang C (2006). Production of an oxidant and SDS-stable alkaline protease from an alkaophilic *Bacillus clausii* I -52 by submerged fermentation: Feasibility as a laundry detergent additive. Enzyme Microbiol. Technol. 38:176–183.

Kim MJ, Dhillon S, Chaudhary, Singh R (1993). Properties of alkaline protease isolated from *Nocardiopsis dassonvillei.* Korean Biochem. J. 26: 81- 85.

Klapper BF, Jameson DM, Mayer RM (1973). The purification and properties of an extracellular protease of *Aspergillus oryzae* NRRL 2160. Biochem. Biophys. 304: 502 – 512.

Kunitz M (1947). Crystalline soyabean trypsin inhibitor II. General properties. J. Gen. Physiol. 30: 291–310.

Lowry OH, Rosebrough NJ, Farr, Randall RJ (1951). Protein measurement with Folin phenol reagent. J. Biol. Chem. 193. 265 – 275.

Manjeet K, Dhillon S, Chaudhary S, Singh R (1998). Production,

purification and characterization of a thermostable alkaline protease from *Bacillus polymyxa*. Indian. J. Microbiol. 38: 63 – 67.
Nasuno S, Onara T (1972). Purification of alkaline protease from *Aspergillus candidus*. Agric. Biol. Chem. 5: 1791 – 1796.
Ng TK, Kenealy WR (1986). Thermostable enzymes and industrial applications. In: (Broch TD ed.). John Wiley and Sons, New York. pp. 197 – 200.
Pandey A, Selvakumar P, Soccol CR, Nigam P (1999). Solid state fermentation for the production of industrial enzymes. Curr. Sci. 77: 149 – 162.
Patel R, Dodia R, Singh SP (2005). Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: Production and optimization Process Biochem. 40: 3569–3575.
Reese ET, Sin RGH, Levinson HS (1950). The biological degradation of cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol. 59: 480 – 485.
Sen S, Satyanarayana T (1993). Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40. Indian J. Microbiol. 33: 43 – 47.
Sinha N, Satyanarayana T (1991). Alkaline protease production by thermophilic *Bacillus licheniformis*. Indian J. Microbiol. 31(4): 425 – 430.
Taguchi H, Hamoki M, Matsuzava H, Ohta J (1983). Heat stable extracellular proteolytic enzyme produced by *Thermus caldophilous* strain GK24 an extremely thermophilic bacterium. J. Biochem. 93: 7 – 13.
Uyar F, Baysal Z (2004).Production and optimization of process parameters for alkaline protease production by a newly isolated *Bacillus* sp. under solid state fermentation. Process Biochem. 39: 1893–1898.
Venugopal M, Saramma AV (2006). Characterization of alkaline protease from *Vibrio fluvialis* strain VM10 isolated from a mangrove sediment sample and its application as a laundry detergent additive. Process Biochem. 41: 1239–1243.
Wang HL, Vespa JB, Hesseltine CW (1974). Production of extracellular proteases by *Aspergillus* species. Appl. Microbiol. 27: 906 – 908.

.