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Optimization and production of Alkaline *Proteases* from Agro byproducts using a novel *Trichoderma Viridiae* strain VPG 12, isolated from agro soil

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ABSTRACT

In recent years, there has been a phenomenal increase in the use of alkaline proteases as industrial catalysts. The aim of this work was to isolate potent fungal strain from the agricultural field of Gulbarga region of India, for the production of alkaline protease by utilizing the agricultural by products *viz*, red and green gram and Bengal gram as substrate under submerged fermentation process. Optimization of fermentation process parameters such as substrate (Red gram husk, green gram husk and Bengal gram husk) utilization, utilization, temperature, pH and incubation period for alkaline protease production was carried out. The maximum production of alkaline protease by Trichoderma VPG 12 was found at pH 8, temperature 35 °C, incubated for 120 h. But the activity of the enzyme could also be seen in a wide range of pH (5-9) and temperature (20-40 °C). With all these properties, the strain can be considered for industrial grade production of alkaline protease.

***Keywords*:** Alkaline proteases; *Trichoderma viridiae*; Agriculture byproduct; Red gram husk

1. INTRODUCTION

The present day trend is the utilization of waste material for production of byproducts, which boosts up high economic returns in many industries[1]. With the advent of biotechnology, attempts have increasingly been made globally to make potential use of agro-industrial residues for value addition by production of enzymes, organic acids, bioactive secondary metabolites, single-cell protein[2].

Alkaline proteases (E.C.3.4.21.14) constitute one of the commercially important groups of extra-cellular microbial enzymes and are widely used in several industrial sectors, particularly in the detergent, food, pharmaceutical, chemical, leather and silk, apart from waste treatment[3]. These enzymes also have potential to contribute in the development of high value added products due to their characteristic nature of aided digestion[4]. Among all proteases, alkaline proteases are robust in nature and are primarily used as detergent additives[5]. They account for 40 % of the total worldwide enzyme sales and this trend is expected to increase in near future[6]. This has created increasing attention in exploitation of exotic microbial strains for production of alkaline proteases.

Red gram ranks sixth among pulses production in world and major legume crop. In India, it is the second largest pulse crop accounting about 20 percent of total pulse production. India annually produces about 2.0-2.5 million tones and stagnant in the past 10 years. The shift in cultivation from pulses to commercial crops and lack of technological innovations to increase yields has hindered the rise in output[7].

The ability of Trichoderma strains to produce extracellular proteases has also been known for a long time; however, the proteolytic enzyme system is relatively unknown in this genus[8]. Fortunately, in the recent years more and more attention is focused on the research in this field. The role of Trichoderma proteases in the biological control of plant pathogenic fungi and nematodes has been demonstrated, and it is suspected that they may be important for the competitive saprophytic ability of green mould isolates and may represent potential virulence factors of Trichoderma strains as emerging fungal pathogens of clinical importance[9].

In view of the above, the present study was aimed for the Proteases production using agro wastes through submerged fermentation. In this study we report on the screening of VPG -12 for its ability of producing proteases using agro wastes such as Red gram, Green gram and Bengal gram husk and their protease production efficacy.

2. MATERIALS AND METHODS

2. 1. Micro-organism

The microorganism used in the present study was *Trichoderma viridae* VPG-12 was isolated from the soil of agricultural waste depositing area and identified in our laboratory on the basis of 16S rDNA sequencing. The 16S rDNA sequence was submitted in NCBI GenBank.

2. 2. Chemicals

Commercially available potato dextrose agar medium was purchased from HiMedia laboratories Pvt. Ltd., India. Lacto phenol Cotton Blue Stain, Casein, Trichloro acetic acid (TCA), sodium carbonate, Folin ceocalteau reagent (FCR) were procured from Merck India, Pvt. Ltd.

2. 3. Fermentation medium

The production medium used was composed for 100 ml of Distilled water was: Casein (40 g), Starch (10 g), Ammonium Chloride (8 g), Calcium chloride (5 g), Sodium phosphate (2 g).

2. 4. Substrate

Red gram husk, green gram husk and Bengal gram husk were collected from Dal-mills and sundried, ground to particle size of 300-500 μm using standard sieves.

2. 5. Inoculum Preparation

Fifty milliliter of pre culture medium consisting of 0.8 % potato dextrose broth was transferred to the cotton- plugged 250 mL Erlenmeyer flask and sterilized in an autoclave for 15 min at 15 Lbs pressure at 121 °C temperature. After cooling at room temperature, the flask was inoculated aseptically with a loopful of fungi from 48 hrs old slant. The flask was then placed in the rotary shaking incubator at 37 °C for 24 hrs.The bacterial growth was used as an inoculum in both the submerged fermentations[10].

2. 6. Production of Alkaline Protease

Submerged fermentation process was employed for production of alkaline proteases[11]. 100 ml of production medium was added to 250 ml Erlenmeyer flask, plugged with cotton, sterilized at 121 °C for 15 min at 15l bs pressure, cooled and inoculated with loopful of fungal strain and incubated at room temperature for 120 hrs under sterilized conditions.

2. 7. Extraction of crude enzymes

After 120 hrs of fermentation period the fermented media was filtered using wattsman's filter paper and centrifuged at 8000 rpm, at 4 °C for 15min and the supernatant was used for analytical studies[12].

2. 8. Assay for alkaline protease

To 0.2 ml of crude enzyme extract, 0.5 ml of 1 % casein and 0.3 ml of 0.2 M phosphate buffer (pH 7) were added and incubated at 60 °C for 10 minutes and the reaction was arrested by addition of 1 ml of 10 % trichloroacetic acid.

The reaction mixture was centrifuged at 8000 rpm for 15 min and supernatant was taken out. To supernatant 5 ml of 0.4 M of Na_2CO_3 and 1ml of 3 fold diluted folin ciocalteau phenol reagent was added and incubated at room temperature for 30 min and the absorbance of the developed blue colour was read at 660 nm.

Using standard tyrosine curve amount alkaline proteases was calculated. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μg of tyrosine substrate (casein) per minute under assay conditions[13].

2. 9. Optimization of fermentation condition

The fermentation process parameters such as pH, temperature, incubation time, different substrates and concentrations that influence production of protease were optimized by varying the conditions.

The experiments were conducted in 250 ml Erlenmeyer flasks containing production medium. After sterilization by autoclaving, flasks were cooled and inoculated with culture maintained under various optimal conditions separately such as pH (5, 6, 7, 8, 9) temperature (20°, 25°, 30°, 35°, 40 °C), fermentation period (24, 48, 72, 96, 120 hrs) and substrates (Casein, Red gram, Bengal gram, Green gram) after 120 hrs of fermentation, the culture filtrate was assayed foe protease activity[14].

2. 10. Standardization of upstream Bioprocess

A batch wise bioprocess was carried out as standard upstream bioprocess for the maximum production of proteases from red gram husk at optimized bioprocess variables such as pH, temperature and fermentation period. In a 250 ml Erlenmeyer flask 100 ml of production medium was taken and sterilized by autoclaving and cooled at room temperature, to this a loopful of culture was inoculated and the process variables such as pH (8), temperature (35 °C) and fermentation period (120 hrs) were maintained and the filtrate was taken for analytical study[15].

3. RESULTS

3. 1. Effect of substrate concentration on protease production

Various agricultural by products rich in protein content were screened for protease production. Among all the substrates, Red gram husk has shown good activity followed by Bengal gram and Green gram.

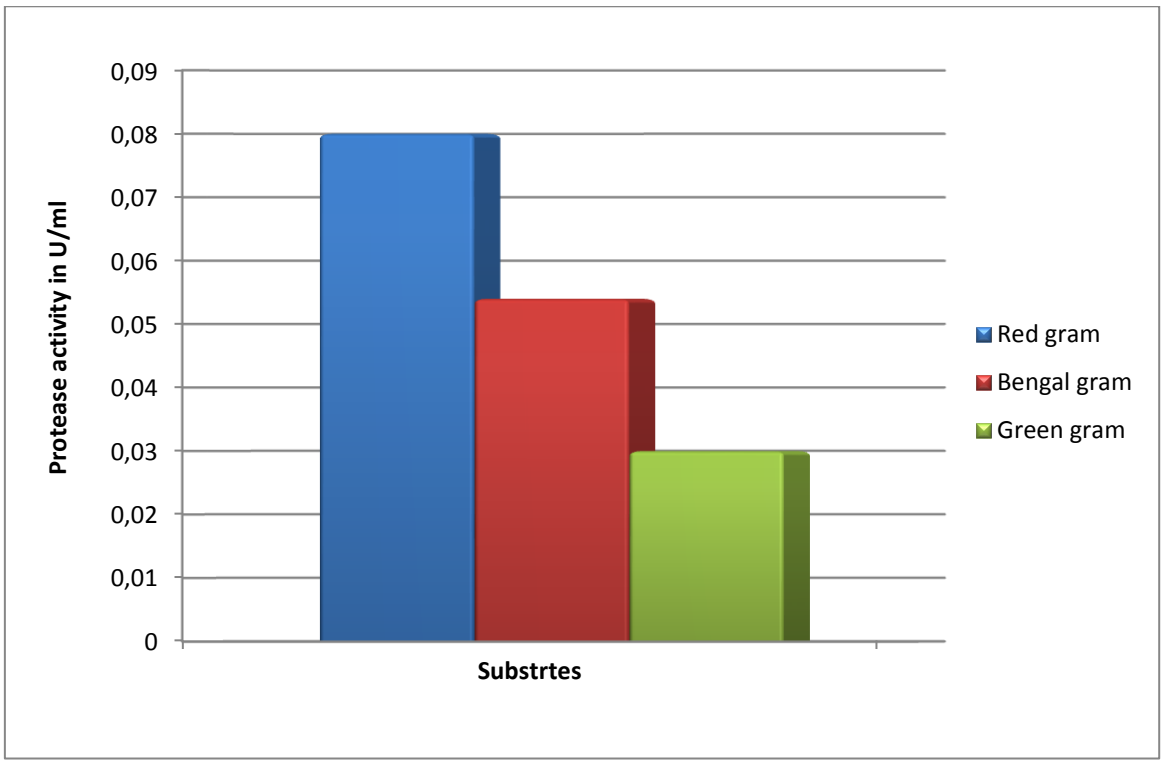

Figure 1. Effect of Substrate concentration on protease production.

3. 2. Effect of pH on protease production

The maximum production of protease in submerged fermentation was observed at pH 8. Figure 2 represents the effect of pH (pH 5-9) on the production of protease.

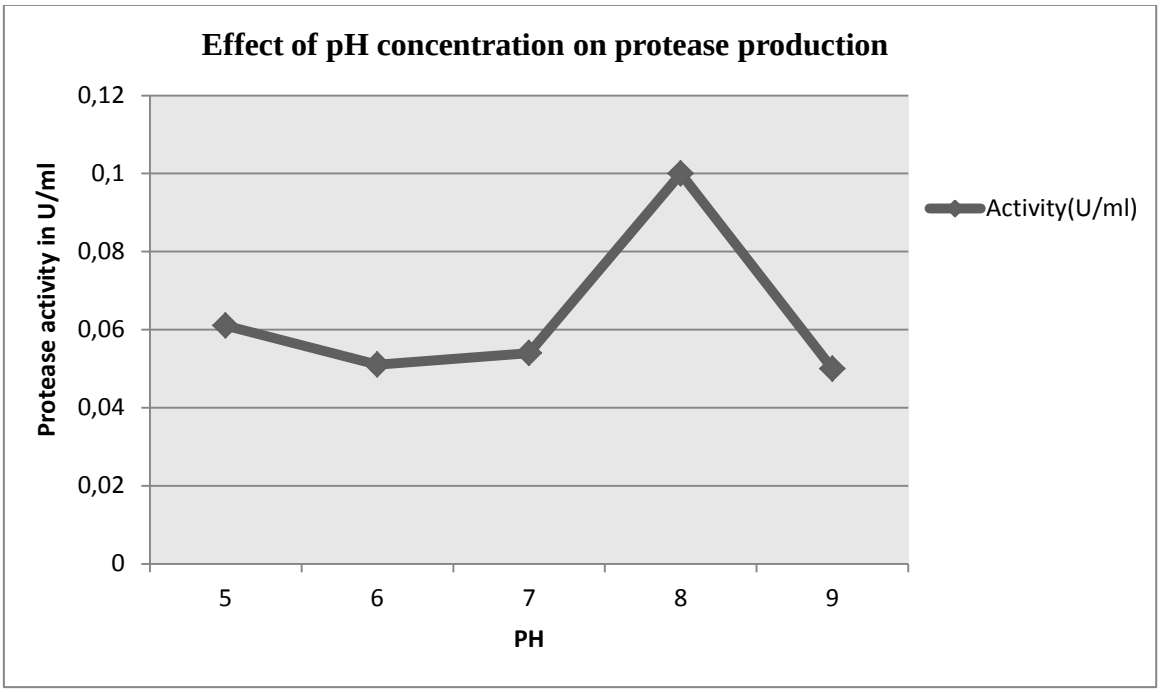

Figure 2. Effect of pH on protease production.

3. 3. Effect of temperature on protease production

The optimum temperature (20-40 °C) for production of protease was depicted in Figure 3. Maximum activity was found at 35 °C and minimum activity was found at 40 °C.

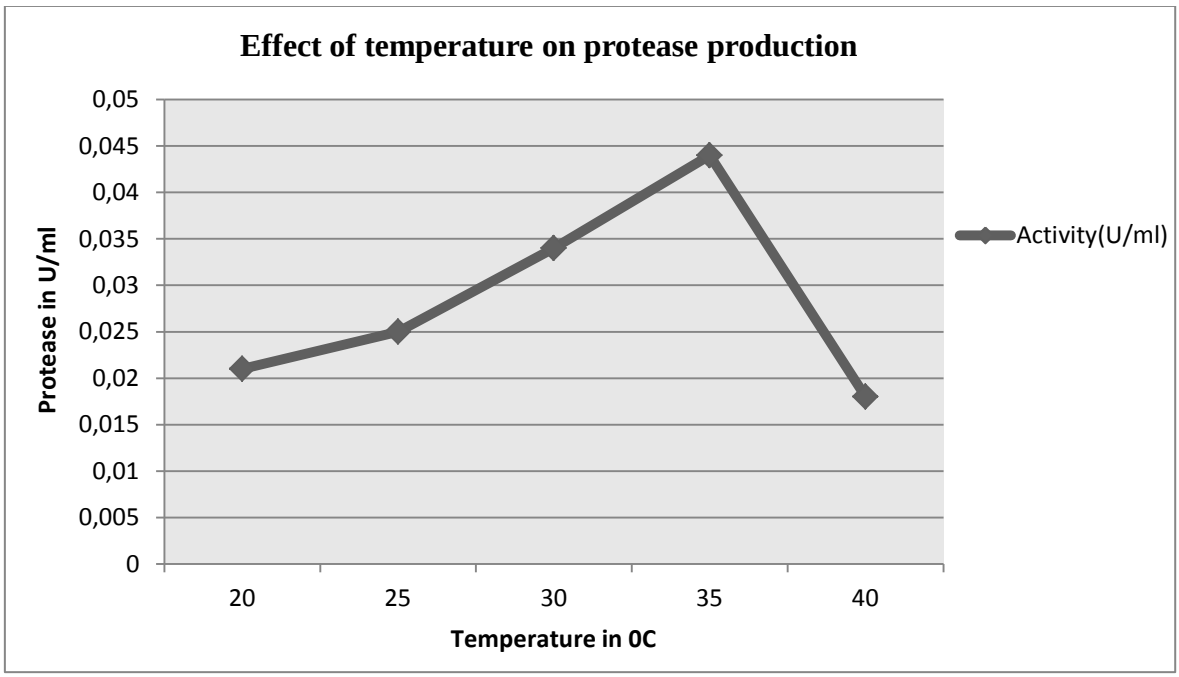

Figure 3. Effect of temperature on protease production.

3. 4. Effect of fermentation period on protease production

Highest yield of protease was observed in 120hrs of fermentation period. The effect of incubation period (24-120 hrs) on the production of protease from submerged fermentation is represented in Figure 4.

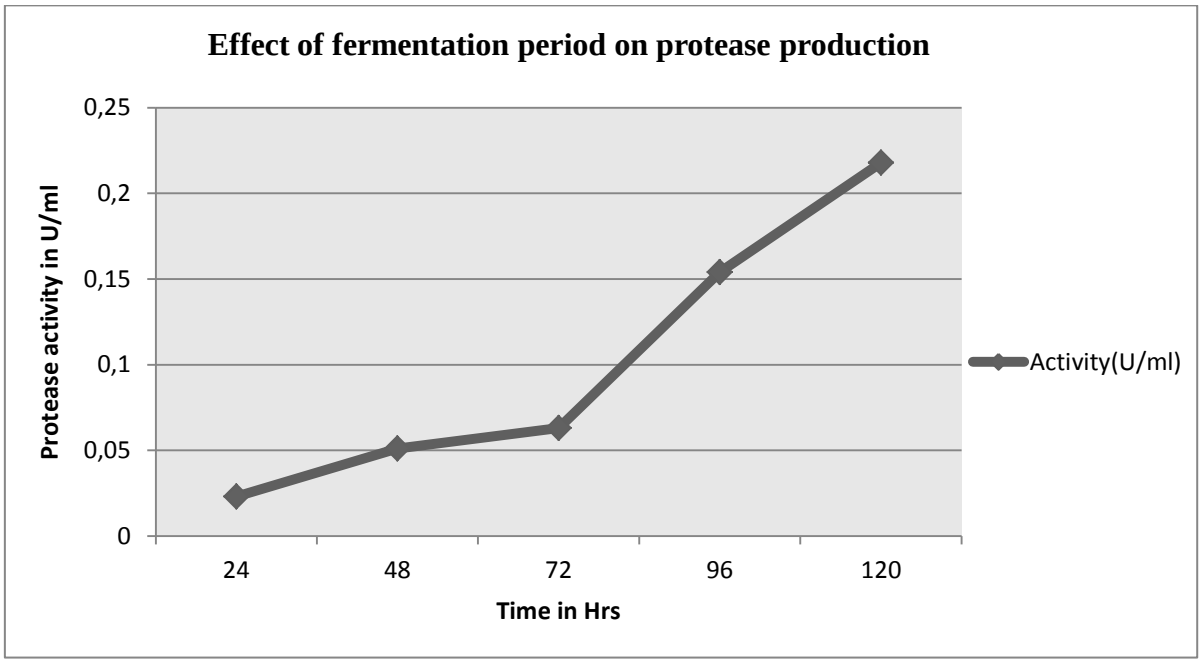

Figure 4. Effect of fermentation period on protease production.

4. DISCUSSION

The selection of an ideal agro industrial byproduct for enzyme production depends on several factors, which are mainly related to cost and availability of the material and thus may involve screening of several such byproducts[16]. During submerged fermentation, different agro industrial byproducts were evaluated for the production of alkaline protease by the organism, and red gram husk, which is a byproduct of Dal mills, was found to be the best protein substrate for the induction of protease production by VPG-12.

The present investigation revels significant valve regarding suitability of Red gram husk as a substrate for production of protease by indigenous strain *Trichoderma* VPG – 12, in submerged fermentation. Many fermenting organisms exhibit satisfactory growth over a broad range of pH with maximum growth at an optimum point[17]. Maximum activity was found at pH 8. The activity was slightly increased from pH 5 to 7; again, yield was reduced at pH 9. Microbial growth rate as with all the chemical reactions is a function of the temperature of 25-35 °C[18]. An attempt was made during the present study to access the influence of range of temperature 20-40 °C and the optimum production of protease was observed at 35 °C.The period of fermentation depends on the nature of media, fermenting organisms and physiological environment. In our study protease, activity was measured from 25 hrs - 120 hrs. The highest activity was found at 120 hrs. The research and development strategies that are employed in finding the substrate and microbes of economic value and for developing their potential in a fermentation process are very crucial and important. The most successful

approach to achieve this object is to understand the multifaceted interaction of the substrate and microorganisms during the course of fermentation. The protease production in standard upstream bioprocess employing Red gram husk in submerged fermentation with regard to efficiency of the strain VPG 12 is ideal in submerged fermentation.

5. CONCLUSION

Findings of the present study support the use of naturally available source for isolation of potent microbes and usage of agricultural by products for production of industrially important enzymes. The isolation and characterization of newer strains of Trichoderma species forever-continuous industrial use from natural sources, where they present in complex mixture is a most powerful means and inevitable. The detection of *Trichoderma VPG12* for production of protease in submerged fermentation is most important contribution of the study.

References

[1] Ashokan P., Mohini S., Shyam R. A., *Building and Environment* 42 (2007) 2311-2320.

[2] Sumantha A., Larroche C., Ashok P., *Food Technol. Biotechnol* 44 (2) (2006) 211-220.

[3] Godfrey T., *Leather in Industrial Enzymology* 2 (1996) 285-291.

[4] McDonald C. E., Chen L. L., *Analytical Biochemistry* 10(1) (1965) 175-177.

[5] Ellaiah P., Srinivasulu B., Adinarayana K., *J Sci Indstr Res* 61 (2002) 690-704.

[6] Gupta R., Beg Q. K., Khan S., Chauhan B., *Appl Microbiol Biotech* 60 (2002) 381-395.

[7] Kalpana Devi M., Rasheedha B. A., Gnanaprabhal G. R., Pradeep B.V., Palaniswamy M., *Ind J Science Tech* 1(7) (2008) 1-6.

[8] Rao M. B., Tanksale A. M., Ghatge M. S., Deshpande V. V., *Microbiol Mol Biol Rev* 62 (1998) 597-635.

[9] Nakayama M., Tomita Y., Suzuki H., Nisizawa K., *J Biochem* 79 (1976) 955-966.

[10] Jeswani L. M., Baldev B., *Adv in Pulse Pro Tech, Indian Council of Agricultural Research Publication* (1988).

[11] Uchikoba T., Mase T., Arima K., Yonezawa H., Kaneda M., *Biol Chem* 382 (2001) 1509-1513.

[12] Manczinger L., Antal Z., Schoop A., Kredics L., *Acta Biol Hung* 52 (2001) 223-229.

[13] Lowry O. H., Rosebrough N., Farr A. L., Ronadall R. L., *J. Biol. Chem* 193 (1951) 265-273.

[14] Nehra K. S., Dhilon S., Chaudhary K., Singh R., *Ind. J. Microbial.* 42 (2002) 43-47.

[15] Joo H. S., Ganeshkumar C., Park G. C., Kim K. T., Seung R., Paik Chang C. S., *Process Biochem* 38 (2002) 155-159.

[16] Gessesse A., *Bioresour. Technol* 62 (1997) 56- 61.

[17] Zwietering M. H., Jongenburger I., Rombouts F. M., Van't Riet K., *Applied and Environmental Microbiology* 56 (1990) 1875-1881.

[18] Malherbe S., Fromion V., Hilgert N., Sablayrolles J. M., *Biotechnology and Bioengineering* 86 (2004) 261–272.

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