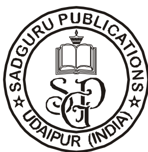

Int. J. Chem. Sci.: 8(2), 2010, 935-942

CRITICAL STUDIES ON KINETIC PARAMETERS FOR THE PRODUCTION OF PROTEASE FROM SSF BY *BACILLUS SUBTILIS* NCIM 2724

D. USHA PRIYANKA, Ch. KANAKA RAJU, A. SUMANJALI K. DWARAKA and V. MEENA*

*Centre for Biotechnology, Department of Chemical Engineering, Andhra University College of Engineering (A), Andhra University, VISAKHAPATNAM – 530003 (A.P.) INDIA

ABSTRACT

Production of protease employing the laboratory isolates of *Bacillus sps.* under solid state fermentation and its kinetic study were the aims of the present study. With the optimized conditions of incubation time 24 hrs, temperature 30^0C, moisture content 40% w/v, inoculum level of 0.8 w/w, and with substrate concentration of 10 g and pH 8.0, glucose concentration 2.0% w/w, the kinetic parameters μ_{max} (0.026 hr^{-1}), yield coefficient $Y_{x/s}$ (0.18) and cell doubling time t_d (20 min) was determined.

Key words: Protease, SSF, Kinetic parameters.

INTRODUCTION

Proteases also known as peptidyl-peptide hydrolysis constitute 60-65% of the global enzyme market[1-3]. The applications of proteases include, in the detergent, food, leather and meat tenderization industries. Proteases are also important tools in studying the structure of proteins and peptides. Besides that, they are also used in pharmaceuticals, medical diagnosis, and decomposition of gelatin on X-ray films as well as in textiles[4-6]. Protease can be produced by several micro-organisms, however, only microbes that produce a substantial amount of extra cellular protease have been exploited commercially. The major proportion of the commercial alkaline protease is derived from *Bacillus* sp.[4,7-9]. The reason for this is their wide temperature, pH tolerance and stability[2].

The current estimated value of the worldwide sales of industrial enzymes is $1

*Author for correspondence; E-mail: meena_sekhar09@yahoo.in

billion of the industrial enzymes; 75% are hydrolytic. Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes[10]. Proteases execute a large variety of functions, extending from the cellular level to the organ and organism level, to produce cascade systems such as haemostasis and inflammation. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions. Their involvement in the life cycle of disease-causing organisms has let them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS[11]. Proteases have a long history of application in the food and detergent industries. Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals is a relatively new development and has conferred added biotechnological importance[12]. The vast diversity of proteases, in contrast to the specificity of their action, attracted worldwide attention in terms of attempts to exploit their physiological and biotechnological applications.

Production of proteases includes several micro-organisms and a variety of substrates. In the present work, papaya peel has been selected as a substrate, which is cheap and commonly available. The main reason in the selection of papaya peel is that it has 57% proteolytic activity[13]. For the commercial exploitation and for the extensive industrial evaluation, it was desirable to optimize the production on large scale using inexpensive media.

EXPERIMENTAL

Materials and methods

Organism: *Bacillus subtilis* (NCIM 2724).

Growth medium

Nutrient agar medium: The selected species of organism were obtained from NCIM – Pune and are maintained on nutrient agar medium at 30^0C for 24 hrs and preserved by sub-culturing every 4 weeks.

Substrate used: Papaya peel.

Chemicals used: Glycine, NaOH, Casein, TCA, Tyrosine, FC reagent.

Instruments used: Digital weighing machine, Autoclave, Laminar air flow chamber, Incubator, UV-VIS spectrophotometer.

Inoculum preparation

The production of proteases requires the preparation of inoculum. Culture was scraped and washed from the slant culture with 10 mL sterile water and 2 mL of this inoculum was added to each 250 mL flasks containing production medium.

Solid state fermentation

10 g of substrate was taken in 250 mL Erlenmeyer conical flasks and to this, 4 mL of water was added. The contents were mixed thoroughly and autoclaved at 121^0C for 15 min. After cooling the flasks to room temperature, the flasks were inoculated with 2 mL of 24 hrs grown culture strain under sterile conditions. The inoculum was prepared by adding sterile distilled water to a 24 hrs old slant. The contents were mixed thoroughly and incubated in a slanting position to provide maximum surface area at 30^0C temperature.

Enzyme extraction

After incubation period, the enzyme was extracted according to the method described by Nagamine *et al*.[14] 50 mL of 0.2 M glycine – NaOH buffer at pH 10 was added and was kept for shaking in an orbital shaker for 30 min. Then the mixture was filtered using a Whattmann No. 1 filter paper. The extracts were collected and then centrifuged. The supernatant was used as enzyme source for protease.

Enzyme assay

Alkaline protease activity was estimated by the modified Auson – Hagihara Method[14]. 1.0 mL of the enzyme solution was added to 6.0 mL of casein (0.6 g of casein mixed in 100 mL of 0.2 M NaOH-glycine buffer) and the mixture was incubated at 37^0C for 10 min. Then 6.0 mL of TCA (Trichloroacetic acid) was added and then incubated for 30 min at room temperature. The mixture was centrifuged for 10 min and the 2 mL of Folin-Ciocalteaue reagent was added to the 1 mL of the supernatant taken and then incubated for 30 min at room temperature. Then the absorbance was read at 660 nm.

RESULTS AND DISCUSSION

Construction of standard graph for tyrosine

Stock solution: It was prepared by dissolving 100 mg of tyrosine in 100 mL of distilled water.

Working solution: To 5 mL of stock solution, 50 mL of distilled water was added.

Procedure

Into a series of test tubes, 0.1 mL, 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, 1.0 mL of standard solution of tyrosine was taken and the volume was made up to 1 mL using distilled water. Into each tube, 5 mL of Na_2CO_3 and 1 mL of F.C. reagent were added and incubated for 30 min at room temperature. Then the absorbance was read at 660 nm. The standard graph was constructed by taking the concentration of tyrosine on X-axis and the O.D values on Y-axis (Fig. 1).

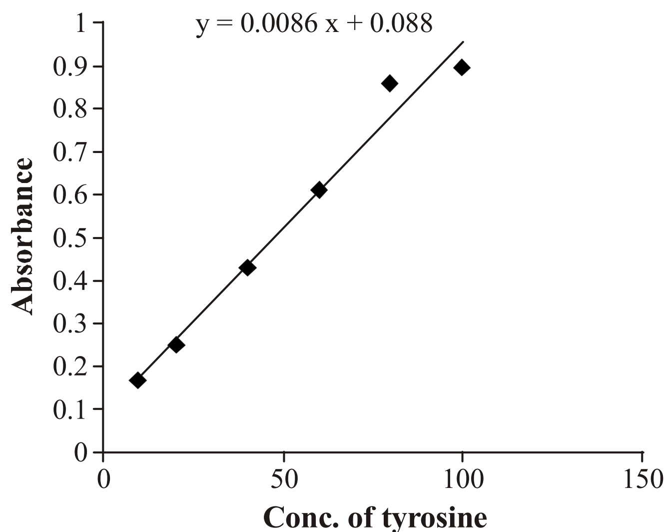

Fig. 1: Standard graph for tyrosine

Effect of kinetic parameters

The effects of maximum specific growth rate, yield factor and doubling time were studied.

Effect of maximum specific growth rate (μ_{max})

The specific growth rate was investigated for the selected *Bacillus* species and it was noticed that with increase in time of fermentation, there is a decrease in substrate concentration and increase in biomass concentration. The maximum specific growth rate was calculated by taking the reciprocal values of specific growth rate and substrate consumption. The maximum specific growth rate for *Bacillus subtilis* was 0.026 hr^{-1}. Results are shown in Fig. 2 as done by Ekram – ul – Haq and Muktar[15].

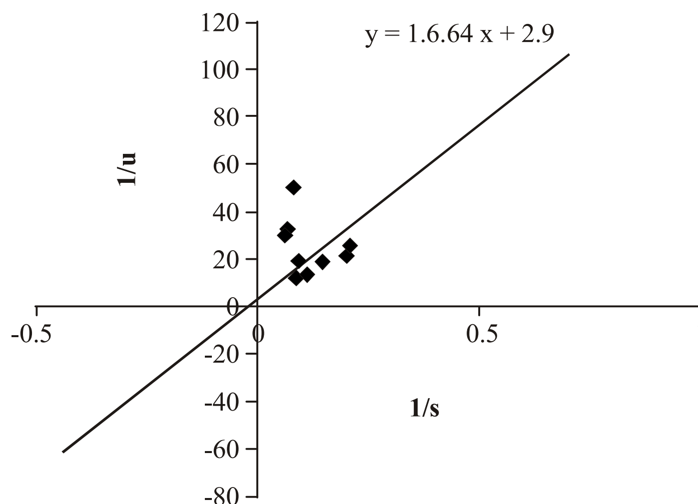

Fig. 2: Effect of specific growth rate

Effect of the yield factor of cell mass ($Y_{x/s}$)

The yield factor ($Y_{x/s}$) is a ratio of amount of biomass formed per amount of substrate consumed. The yield factor investigated on the selected *Bacillus* species was 0.18 mg/mL. This was determined by the plot between $r_{x/x}$ vs . $r_{s/x}$. The slope of the graph gives yield factor. 2 hrs of incubation time interval was selected for determining the yield factor ($Y_{x/s}$) for the *Bacillus* species (Fig. 3). Results are based on the work by Ekram–ul – Haq and Muktar[15].

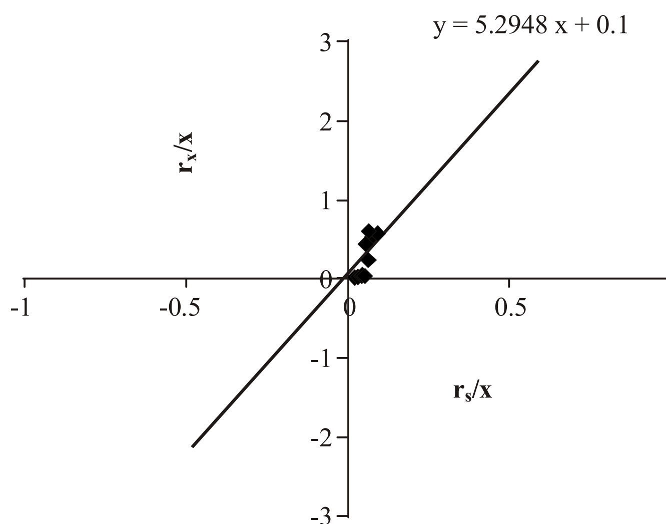

Fig. 3: Effect of yield factor ($Y_{x/s}$)

Effect of cell doubling time

The time required for the microbial mass to double is called doubling time. The cell doubling time was investigated on the selected *Bacillus* species. It was determined during the exponential growth of the microbe, characterized by a straight line on a semi-log graph of ln x vs. time. The cell doubling time for the selected *Bacillus* species is 20 min. The results are shown in the Fig. 4.

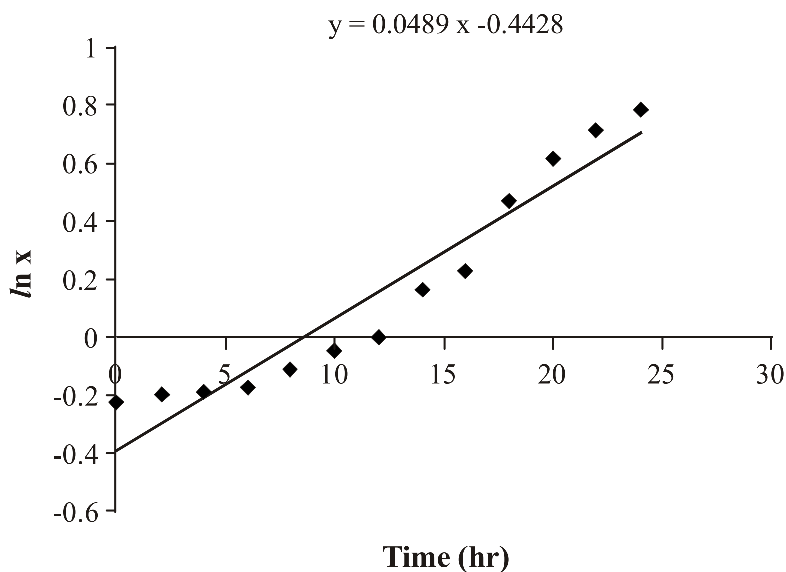

Fig. 4: Effect of doubling time

CONCLUSIONS

The growth kinetic parameters for *bacillus subtilis* using monod kinetics for the production of protease was studied. The specific growth rate (μ_{max}), yield factor Yx/s and the doubling time for *Bacillus subtilis* was found to be 0.026 hr^{-1}, 0.18 mg/mL and 20 min, respectively. It was observed that even after 24 hrs, the biomass and also the production of the enzyme remained constant, which indicates that the organism has already reached the stationary phase and even after sometime, there was a decrease in the biomass, which indicates that the organism has reached the death / decline phase. From the obtained results, it has been concluded that 24 hours was the optimized time for the production of protease by *bacillus subtilis*.

REFERENCES

1. C. V. Banerjee, R. K. Sani, W. Azmi and R. Soni, Thermostable Alkaline Protease from *Bacillius Brevis* and is Characterized as a Laundry Determine Additive, Process Biochem., **35**, 213 – 219 (1999).
2. H. Genckal, and C. Tari, Alkaline Protease Production from Alkalophilic *Acillus Sp*. isolated from Natural Habitats, Enzyme Microbial Technol., **39**, 703-710 (2006).
3. R. S. Laxman, A. P. Sonawane, S. V. More, B. S. Rao, M. V. Rele, V. V. Jogdand, V. V. Deshpande and M. B. Rao, Optimization and Scale up of Production of Alkaline Protease from Conidiobolus Coronatus. Process Biochem., **40**, 3152 – 3158 (2005).
4. H. S. Joo, C. G. Kumar, G. C. Park, K. M. Kim, S. R. Paik and C. S. Chang, Optimization of the Production of an Extracellular Alkaline Protease from *Bacillus Horikoshii*, Process Biochem., **38**, 155 (2002).
5. R. Patel, M. Dodia and S. P. Singh, Extracellular Alkaline Protease from a Newly isolated Haloalkaliphilic *Bacillus Sp*. Production and Optimization, Process Biochem., **40**, 3569 – 3575 (2005).
6. C. Tari, H. Genckal and F. Tokatali, Optimization of a Growth Medium using a Statistical Approach for the Production of Alkaline Protease from a Newly isolated *Bacillus sp*. L 21, Process Biochem., **41**, 659 – 665 (2006).
7. J. K. Yang, I. L. Shih, Y. M. Tzeng and S. L. Wang, Production and Purification of Proteases from a *Bacillus Subtilis* that can Deproteinize Crustacean’s Wastes, Enzyme Microbial Technol. (2000).
8. R. S. Prakasham, Ch. Subba Rao and P. N. Sarma, Green Gram Husk, An Inexpensive Substrate for Alkaline Protease Production by *Bacillus Subtilis* in Solid State Fermentation, Elsevier Ltd. (2005).
9. D. R. Durham, D. B. Steward and E. J. Stellw, Novel Alkaline and Heat Stable Serine Protease from *Bacillus Sps*. EX6638. J. Bacteriol., **169**, 2762 – 2768 (1987).
10. E. M. El–Safey and U. M. Abdul Raouf, Production, Purification and Characterization of Protease Enzyme from *Bacillus subtilis*.
11. Valeria F. Soares, Leda R. Castille, Elha P. S Bon and Denise, M. G. Freire, High Yielding *Bacillus subtilis* Protease Production by Solid State Fermentation, Humana Press, (2005) pp. 311- 319.

12. R. S. Pagare, A. M. Ramdasi, S. R. Khandelwal, M. O. Lokhande and B. A. Aglave, Production and Enzymatic Activity of an Extracellular Protease from *Aspergillus Niger* and *Bacillus Subtilis*, Int. J. Biotechnol., **4** (2008).

13. Rubens Monti, Carmelita A Basilio, Hemique C. Trevision and Jonas Contiero. Purification of Papain from Fresh Latex of *Carica Papaya*, Braz. Arch. Boil. Technol., **43** (2000).

14. P. Ellaiah, B. Srinivasulu and K. Adinarayana, A Review on Microbial Alkaline Proteases. J. Sci. Ind. Res., **61**, 690 – 704 (2002).

15. Ekram-Ul-Haq and Hamid Muktar, Biosynthesis of Proteses from *Lacto Bacillus Paracasei*; Kinetic Analysis. Indian J. Biochem. & Biophys., **43**, 377-381 (2006).

16. B. Hagihara, H. Matsubara, M. Nakai and K. Kunuki, Preparation of Crystalline Proteinase of Bacillus Subtilis, J. Biochem., **45**, 185 – 194 (1958).

17. Sigma Product Information, Sigma Quality Control Test Procedure, Enzymatic Assay of Protease.

18. O. I. Oloyede, Chemical Profile of Unripe Pulp of Carica Papaya, Pak. J. Nutrition, **4(6)**, 379 – 381 (2005).

Accepted : 17.11.2009