Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

Asian Journal of Plant Science and Research, 2012, 2 (3):376-382

ISSN : 2249-7412
CODEN (USA): AJPSKY

Production, Purification and Characterization of α-Amylase and Alkaline Protease by *Bacillus* sp. HPE 10 in a Concomitant Production Medium

Mukesh kumar D J[1]*, Andal Priyadharshini D[2], Suresh K[2], Saranya GM[2], Rajendran K[2] and Kalaichelvan PT[1]

[1]*Centre for Advanced Studies in Botany, University of madras, Guindy Campus, Chennai, TN, India*
[2]*A.V.V.M. Sri Pushpam College (Autonomous), Poondi, Thanjavur Dist, TN, India*

ABSTRACT

This study evaluated an amylolytic and proteolytic bacterial strain HPE 10 isolated from soil sample. Initial screening process involved a selection medium containing starch and skim milk as substrates. The strain was characterized as Bacillus sp. using Bergey's manual and 16S rDNA sequence analysis. Various physico-chemical parameters such as pH, temperature, incubation period, and effect of different carbon and nitrogen sources were studied in order to determine the optimum conditions for amylase and protease production by Bacillus sp. Amylase production was influenced by 120 h of incubation, 50°C with initial pH of 6 along with lactose and sodium nitrate. Protease production was influenced after 120 h of incubation, 45°C, pH 9 with glucose and peptone as nutrient sources. The enzymes were purified by column chromatography and molecular weights were determined by SDS-PAGE.

Keywords: *Bacillus*, amylase, protease, lactose, sodium nitrate, glucose, peptone.

INTRODUCTION

Enzymes are biological catalysts which regulate specific biochemical reactions. Among the industrially important enzymes, proteases and amylases are considered to be the most prominent enzymes since they are widely utilized in brewing, detergent and food industries. There is difference in the properties of amylases and proteases produced by various bacterial strains with reference to temperature, pH etc., [1]. These enzymes have more advantages in detergent formulations with varying temeperature and pH. Proteases and amylases are the first and second enzyme classes used in the formulation of enzymatic detergent [2].

The source of the enzymes includes plants, animals and microbes. Of that microbial source were mostly preferred since they are easy to handle. Among the microbial sources, bacteria especially *Bacillus* sp. were found to be predominant in the production of these enzymes due to their shorter generation time, and the ease of genetic and environmental manipulation [1]. Selection of a suitable strain capable of producing both amylase and protease together with commercially acceptable yield is a crucial step for bioprocessing [3].

The production of these enzymes is very important since they account 70% of global industrial enzyme market [4]. In addition, production of both the enzymes in one-step increases the efficiency economy of the process. It is well known that enzyme production by microorganisms is greatly influenced by media components, especially carbon and nitrogen sources and the physical factors such as incubation time, pH and temperature [5].

The present study focused towards two important aspects: The production of two economically important enzymes namely amylase and protease in a combined production medium. Further optimization of environmental factors and media components on amylase and protease production using a one-factor at a time approach.

MATERIALS AND METHODS

Sampling Site

Effluent sample was collected from the Henkel India Ltd., Ambattur, Chennai (latitude 13°06′36″N and longitude 80°10′12″E) in clean containers. The sample was transferred to laboratory and stored at 4°C.

Sample Processing

The effluent from the detergent industry was heated at 65 °C for 30 min in order to eliminate thermolabile bacteria. The heat treated sample was further diluted to minimum concentration and plated on nutrient agar (NA) plates.

Screening for Enzyme Production

The isolated colonies grown on NA plates were screened for their ability to produce α-amylase by streaking them on Starch agar plates (soluble starch 1.0%, yeast extract 0.4%, K_2HPO_4 0.1%, and $MgSO_4 \cdot 7H_2O$ 0.15%, pH 7.0) [6]. After 24 h incubation, the amylase producers were identified by flooding the plates with 1% iodine solution. The typical colonies showing clear zones were selected and further screened for proteolytic activity

The proteolytic activity of the amylase positive strains were screened by streaking them on skim milk agar plates (Skim milk 1.0%, Peptone 0.1%, NaCl 0.5%, Agar 2.0% and pH 10.0) [7]. The protease positive strains were selected by the formation of clear zones. The strain showing positive for both the enzymes with maximum activity was selected based on zone diameter and proceeded for further production and characterization purposes.

Morphological Characterization and Identification

The isolate was characterized morphologically by Bergey's manual of systemic bacteriology [8]. The authenticated identification was done by 16S rDNA analysis. The 16S rDNA gene was amplified by PCR using primers: 63f (5′-CAGGCCTAACACATGCAAGTC) and 1387r (5′-GGGCGGWGTGTACAAGGC) [9]. The DNA was sequenced (Chromous Biotech, Chennai) and BLAST analysis was conducted (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Enzyme Production

The test isolate was inoculated in the concomitant production medium (soluble starch 0.25%, whey protein concentrate 0.01%, corn steep liquor 0.03%, KCl 0.03%, $MgSO_4$ 0.05%, K_2HPO_4 0.087%, $CaCl_2$ 0.00022%, ZnO 0.00025%, $FeCl_3.6H_2O$ 0.0027%, $MnCl_2.4H_2O$ 0.001%, $CuCl_2.2H_2O$ 0.000085%, $CoCl_2.6H_2O$ 0.00024%, $NiCl_3.6H_2O$ 0.000025%, H_3BO_3 0.00003, Peptone 0.1) as described by Thamy et al. [10]. The pH was adjusted to 7.5 and incubated in a shaker at 120 rpm at room temperature for 96 h. After incubation, the samples were withdrawn and centrifuged at 4500 × *g* for 15 min. The supernatant was used as the crude enzyme preparation.

Amylase Activity

The amylolytic activity was assayed by measuring the reducing sugar released when starch was used as substrate. The reaction mixture containing 2.0 ml of the crude enzyme, 2.0 ml of 0.1 M acetate buffer and 1% soluble starch (pH 7.0) was incubated at 37 °C for 30 min. The amount of reducing sugar released was determined by the addition of 3,5-dinitrosalicylic acid followed by boiling for 10 min according to Bernfield [11]. The absorbance was read at 540 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar in one minute under the assay condition.

Protease Activity

The proteolytic activity was assayed by modified folins method [12]. The reaction mixture comprises 0.5% Casein and 50 µM Glycine NaOH buffer (pH 10.0) was kept for incubation at 80 °C for 30 min. The reaction was then stopped by the addition of 10% TCA. The tyrosine liberated was estimated using Folins reagent and absorbance was recorded at 670 nm. One unit of protease was defined as amount of protease required to release 1 µg of tyrosine under the assay condition in 1 min.

Biomass
The biomass of the bacterial culture was read spectrophotometrically at 600 nm [13].

Total Protein Content
The total protein content in the crude enzyme was analysed by Bradford's method [14].

Optimization of Culture Conditions
The various operating variables for fermentation were optimized which included pH, temperature, time of incubation and inoculum concentration. Optimal temperature and pH were obtained by varying the temperature range from 35 °C to 65 °C and pH 5.0 to 11.0. In addition, production of enzymes was monitored from 24 to 144 h of incubation. The optimized culture conditions were used for in vitro enzyme production.

Effect of Carbon and Nitrogen Source
Different carbon sources such as sucrose, lactose, glucose, starch, fructose and mannase were incorporated into the basal medium by replacing the carbon source in the production medium to analyze its role on the production of both the enzymes. Similarly, the enzyme production was optimized by different nitrogen sources such as yeast extract, beef extract, peptone, ammonium chloride, sodium nitrate and ammonium carbonate incorporated in the basal medium.

Enzyme Extraction and Purification
The crude enzyme produced was subjected to ammonium sulphate precipitation to remove the unwanted protein components (30% saturation for α-amylase whereas 75% saturation for alkaline protease).

The α-amylase was purified by the method proposed by Krishnan and Chandra [15]. The precipitated α-amylase was dissolved in 0.01 M Sodium phosphate buffer (pH 6.4). The elution was done using Carboxymethyl cellulose column pre-equilibrated with the same buffer under a linear gradient of NaCl. Flow rate was adjusted to 40 ml per hour and 200 ml fractions were collected and assayed for amylolytic activity. The fractions with maximum activity was pooled, concentrated and stored at 4 °C.

The alkaline protease was purified using ion exchange chromatography [16]. The precipitated enzyme was subjected to ion exchange chromatography with DEAE Sephadex A-50 column, 50 mM phosphate buffer (pH 7.0) as Running buffer and 1M NaCl in 50mM phosphate buffer as elution buffer (pH 7.0). The fractions with maximum proteolytic activity was collected, pooled, concentrated and stored for future uses.

SDS-PAGE Analysis
The separation was done in denaturing conditions [17] on 12% polyacrylamide gel. Samples in 20 µl quantities were loaded into electrophoretic wells and electrophoresis done at room temperature using a constant current of 200 mA per gel for 2 hours. The standard marker was used for reference purpose and Coomasie brilliant blue staining was done for band visualization.

RESULTS AND DISCUSSION

Bacillus sp. HPE 10 isolated from soil was found as an effective producer of amylases and proteases evident from the findings. The enzyme together helps in the stain removal having varied applications in detergent industries [3]. A total of 27 bacterial isolates were obtained from soil samples. They were initially screened for amylase production based on zone of clearance on starch agar plates. Among the 27 isolates, eight of them had the ability to produce amylases which were further screened for protease production in skim milk agar plates. The best producer of both amylase and protease enzymes were further selected for optimization studies.

While characterizing the bacteria based on Bergey's manual of systemic bacteriology, the isolated strain was found to be gram positive, rod shaped, spore forming, and mobile bacterium. The organism was further identified using 16S rDNA methodology. The 16S rDNA analysis helps in the identification of *Bacillus marini* which was isolated from Andaman [18]. Part of the 16S rDNA sequence was amplified and sequenced. Comparing the obtained sequence with the sequences available in NCBI, the bacterial strain revealed similarity with the *Bacillus* sp. and submitted to Genbank database with the accession number of JQ904294 as *Bacillus* sp. HPE 10. The *Bacillus* sp. are

found to be having prestigious place in enzyme production technology as it serves as the source for various enzymes [19].

The production of both the enzymes started in exponential phase and the activity of the enzymes showed linearity with the growth of the tested strain. The maximum production of protease was obtained at 72 h and remained stable until 120 h. In the same medium, the maximum amylase activity was attained after 120 h (Fig. 1). The production of proteases usually lies between 24 to 120 h [20, 21]. In contrast the amylase production attained its maximum by 96 h reported by Ashwini et al. [18].

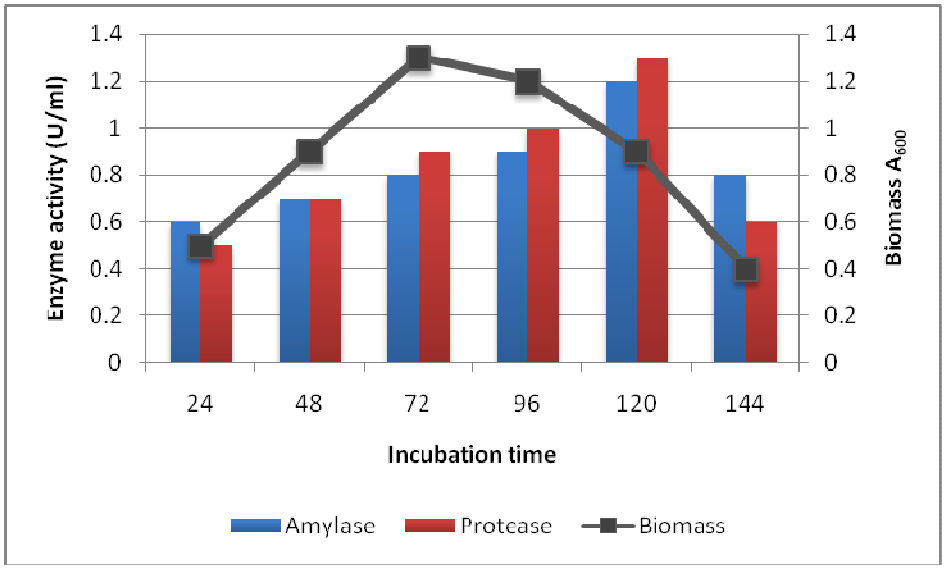

Fig. 1: Enzyme activity and growth profile with respect to different incubation time

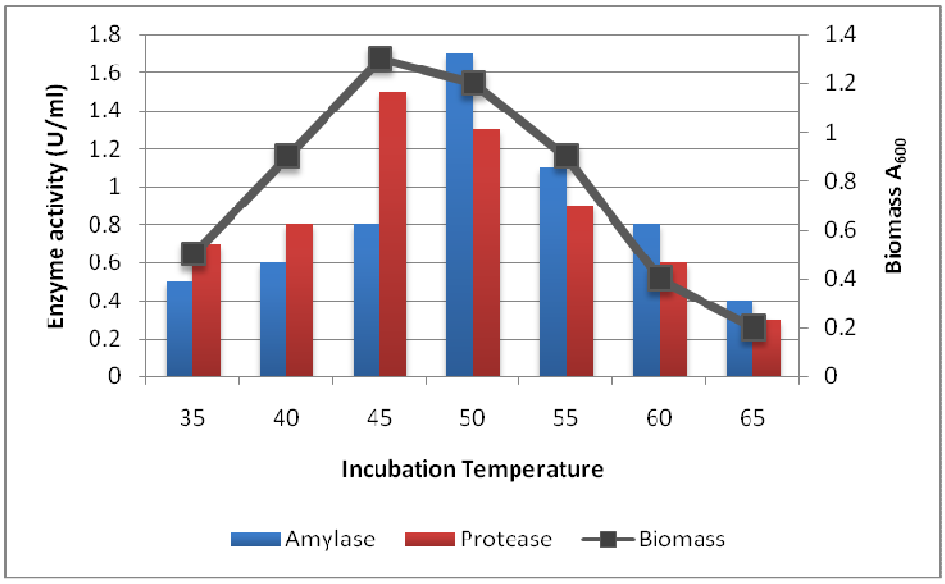

Fig. 2: Enzyme activity and growth profile with respect to different temperature

The initial pH and incubation temperature are the most critical factors that have to be optimized for enzyme production. The optimum temperature for protease production by *Bacillus* sp. was found to be 45 °C and beyond which the enzyme activity was reduced gradually (Fig. 2). The amylase activity attained its maximum at 50°C.

Protein content was also found to be maximum at this temperature. From the observed results, the isolated strain was found to be mesophilic. The initial temperature of 35 °C [22] and 40 °C [18] was found to be optimum for the production of alkaline protease and amylase respectively.

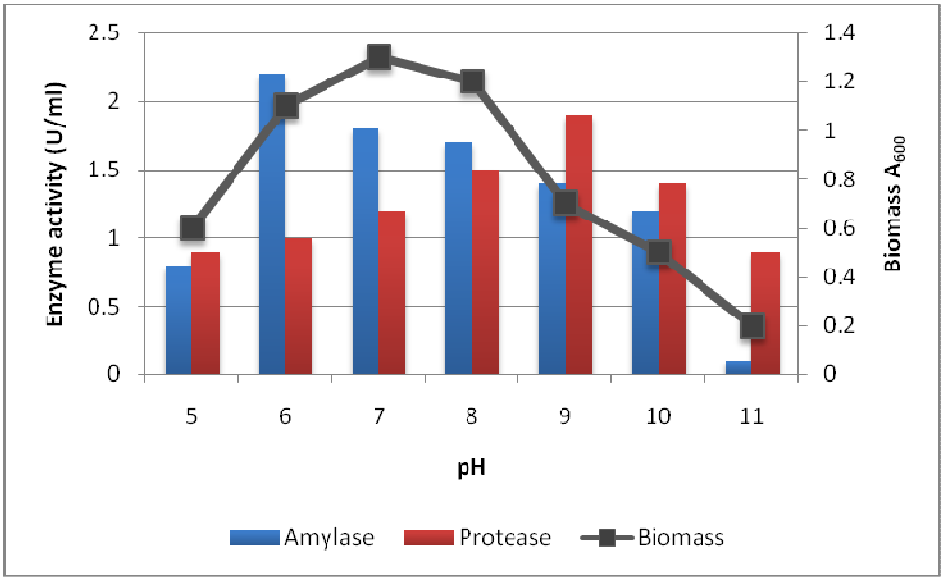

Fig. 3: Enzyme activity and growth profile with respect to different pH

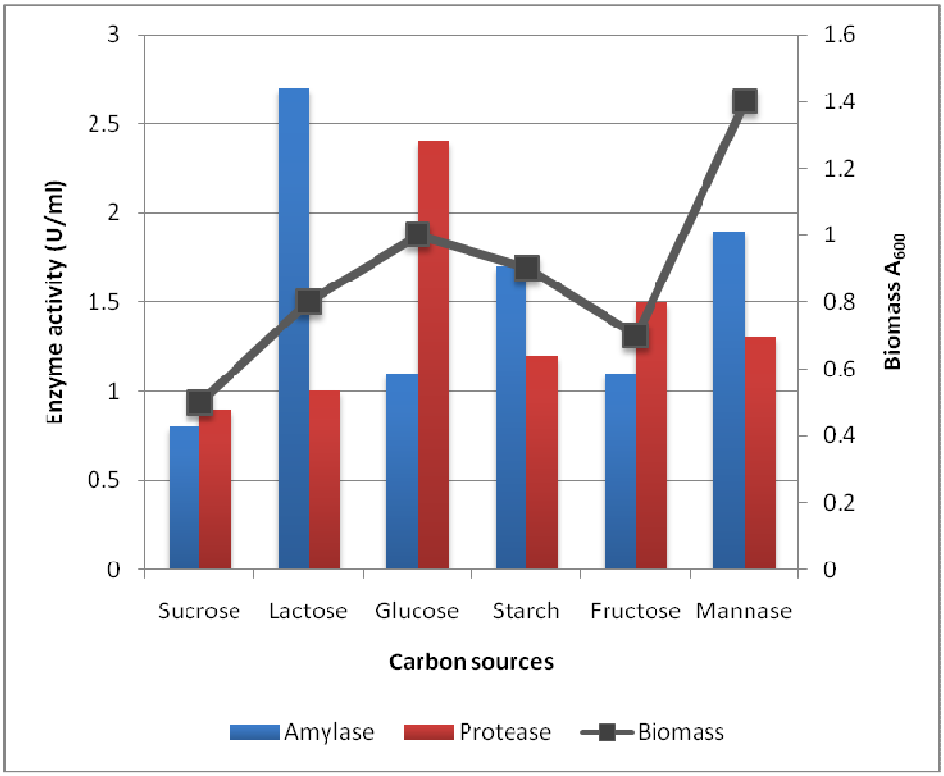

Fig. 4: Enzyme activity and growth profile with respect to different carbon sources

The effect of the initial pH of the production medium was tested at varying pHs 5–11. Considerably less production of enzymes was obtained at acidic pH and comparably increased enzymes production was obtained at weak alkaline pH (Fig. 3). The maximum production of protease was obtained at pH of 9.0. At pH of 7.0, high amylase activity was obtained. The results indicated that the isolated *Bacillus* sp. was a alkaline organism. The weak alkaline conditions were found to be optimum for the production of both the enzymes [18, 22].

The production of enzyme is greatly dependent on the condition of growth of the culture and composition of nutrient medium. The medium components have been predicted to play a significant role in enhancing the enzyme production [23]. Various carbon sources were used for the production of amylase and protease by the isolated *Bacillus* sp., Glucose was found to be the best carbon source for protease production whereas disaccharide lactose favored amylase production. The increase in enzyme production was parallel to the protein content (fig. 4).

Bacillus sp. was allowed to grow in the presence of different (organic and inorganic) nitrogen sources, replacing the total nitrogen sources from the production medium with equivalent amount of nitrogen. Among the organic nitrogen sources used, sodium nitrate had significant effect on extracellular amylase production (Fig. 5). Interestingly maximum proteolytic activity was achieved, when the cells were grown in a medium containing peptone. Poor amylase enzyme secretion was observed in the medium containing ammonium chloride. Simple inorganic nitrogen sources like ammonium chloride and sodium nitrate did not support the protease production. The findings revealed that not all carbon and nitrogen sources acted as enhancer for combined production of the enzymes in a single production system.

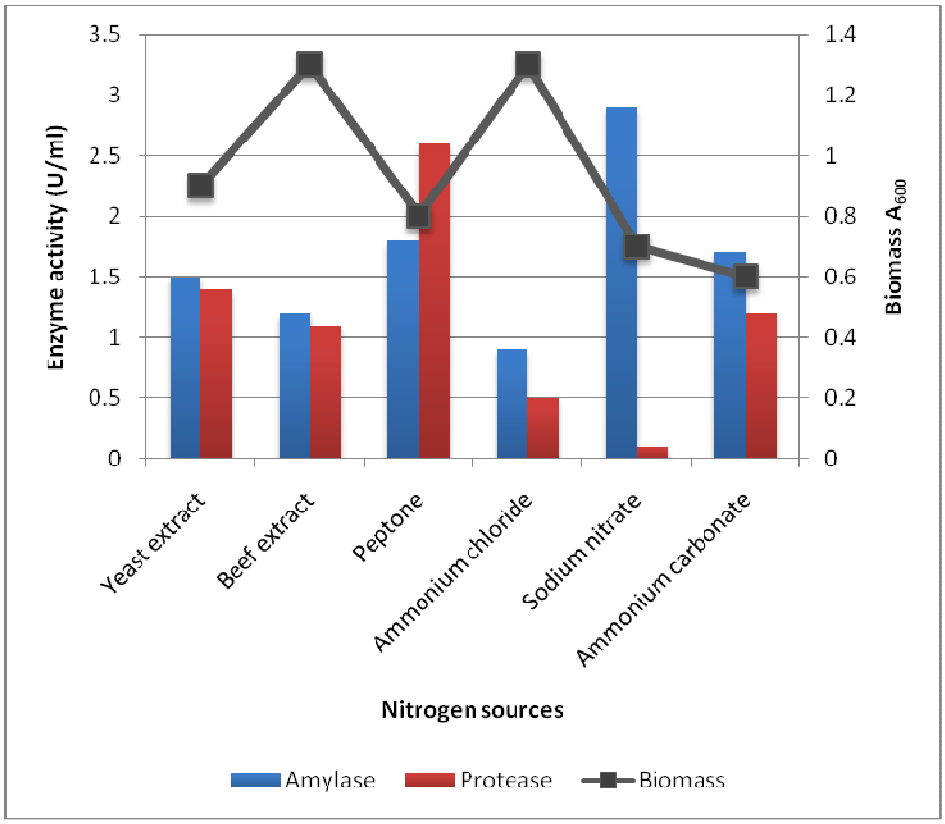

Fig. 5: Enzyme activity and growth profile with respect to different nitrogen sources

The enzymes produced under optimal conditions were purified by ammonium sulphate precipitation and ion exchange chromatography. The alkaline proteases and amylases are positively charged and cannot be bounded with anionic exchangers. Hence, in the study, a cationic exchanger, DEAE Sephadex was used for the purification of alkaline protease and CMC column for amylase.

Among the various fractions collected from CMC column, the fractions with maximum enzyme activities were selected and analyzed by SDS-PAGE. The presence of single band near the 30 kDa marker protein was confirmed as amylase.

The purified protease was checked for its homogeneity and molecular weight was determined by SDS-PAGE. The purified protease migrated as a single band corresponding to molecular mass of 30 kDa. The findings are in accordance with Beg et al. [24], Johnvesly and Naik [25] and Singh et al. [26].

CONCLUSION

A novel *Bacillus* sp. HPE 10 was isolated from soil with the ability of producing amylase and protease enzyme together was studied in detail. The culture conditions and media components were optimized for better production of both the enzymes. It is important to produce both the enzymes together as they have combined application in detergent formulation. The production medium was found to be cost effective, convenient and easier to scale up than the conventional method of blending enzymes. Further experiments will be carried out to obtain high yield of amylase and protease.

REFERENCES

[1] R. Gupta, P. Gigras, H. Mohapatra, V.K. Goswami, Chauhan B, *Process Biochem.* **2003**, 38, 1599–1616.
[2] S. Mitidieri, A.H. Souza Martinelli, A. Schrank, M.H. Vainstein, *Bioresour. Technol*., **2006**, 97, 1217–1224.
[3] S. Negi and R. Banerjee, *Afr. J. Biochem. Res.*, **2010**, 4(3), 73–80.
[4] C. W. Merheb, H. Cabral, E. Gomes, R. DaSilva, *Food Chem.*, **2007**, 104(1), 127–131.
[5] M. Cotârleț, G. E. Bahrim, *Turk. J. Biochem.*, **2011**, 36(2), 83–92.
[6] T. Beffa, M. Blanc, L. Marilley, J. Lott Fisher, P.F. Lyon, Taxonomic and metabolic microbial diversity during composting. In: The Sciences of Composting, M. De Bertoldi, P. Sequi, B. Lemmes, T. Papi, Eds., (Blackie Academic and Professional: Glasgow, UK, **1996**) 149–161.
[7] A. S. S. Ibrahim and A. I. Eldiwany, *Aust. J. Basic Appl. Sci.*, **2007**, 1(4), 473–478.
[8] H.A. Sneath, M.S. Peter, H. Nicolas, G.L. Hold, Bergey's manual of systemic bacteriology (vols. 1 & 2) (Williams and Wilkins Company, Baltmore, MS, USA), **1986**.
[9] J.R. Marchesi, T. Sato, A.J. Weightman, T.A. Martin, J.C. Fry, S.J. Hiom, D. Dymock and W.G. Wade, *Appl. Environ. Microbiol*., **1998**, 64, 795–799.
[10] Thamy Lívia Ribeiro Corrêa, Stella Karla dos Santos Moutinho, Meire Lelis Leal Martins, Marco Antônio Martins, *Ciênc. Tecnol. Aliment.*, **2011**, 31(4), 843-848
[11] Bernfield P, Methods in Enzymology, Vol. 1, (Academic Press: New York, USA) **1955**, 149–158.
[12] Amit Verma, Hukum Singh Pal, Rachna Singh and Sanjeev Agarwal, *Int. J Agric. Environ. Biotechnol.*, **2011**, 173–178
[13] C. Henroette, S. Zinebi, M.F. Aumaitre, E. Petitdemange, H. Petitdemange, *J. Ind. Microbiol.*, **1993**, 12: 129-135.
[14] M.M. Bradford, *Anal. Biochem.*, **1976**, 72, 248–254.
[15] T. Krishnan and A.K. Chandra, *Appl. Environ. Microbiol.*, **1983**, 46(2), 430–437.
[16] H. Ariffin, N. Abdullah, M.S. Umi Kalsom, Y. Shirai and M.A. Hassan, *Int. J. Eng. Technol.*, **2006**, 3(1), 47–53.
[17] U.K. Laemmli, *Nature*, **1970**, 227, 680–685.
[18] K. Ashwini, K. Gaurav, L. Karthik, K.V. Bhaskara Rao, *Arch. Appl. Sci. Res.*, **2011**, 3(1), 33–42.
[19] It Jamilah, Anja Meryandini, Iman Rusmana, Antonius Suwanto, and Nisa Rachmania Mubarik, *Microbiol. Indones.*, **2009**, 3(2), 67–71.
[20] Q.K. Beg and R. Gupta, *Enzyme Microbiol. Technol.*, **2003**, 32, 294–304.
[21] K. Krishnaveni, D.J. Mukesh Kumar, M.D. Balakumaran, S. Ramesh and P.T. Kalaichelvan, *Der Pharm. Lett.*, **2012**, 4(1), 98–109.
[22] Fikret Uyar, Ilknur Porsuk, Göksel Kizil, Ebru Ince Yilmaz, *Eur. J. BioSci*., **2011**, 5, 1–9.
[23] R. Gupta, Q.K. Beg, S. Khan, B. Chauhan, *Appl. Microbiol. Biotechnol*., **2002**, 60, 381–395.
[24] Q.K. Beg, R.K. Saxena, R. Gupta, *Biotechnol. Bioeng.*, **2002**, 78, 289–295.
[25] B. Johnvesly, G.R. Naik, *Process Biochem*., **2001**, 37, 139–144.
[26] J. Singh, N. Batra, R.C. Sobti, *Process Biochem.*, **2001**, 36, 781–785.