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Microbial Utilization of Potato Wastes for Protease Production and Their Using as Biofertilizer

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Abstract: Production of proteases, related to various industrial applications, especially the waste after fermentation was testes as a biofertilizer, was done from a selected yeast *Saccharomyces serevisiae*. Enzymes yield was maximized by optimizing the composition of a low-cost culture medium. Maximum production of enzyme (360 U/mg protein) was obtained at medium containing 15 g substrate, initial pH 6.0, after fermentation at 20 °C for 72 h. Ammonium sulfate was good nitrogen sources for enzymes production. The largest proteases activities was obtained in 15 g solid potato waste with Maltose as carbon source. Application of biologically treated agro-wastes was able to increase plant development, the addition of potato waste to cultivated soil without treatment with *S. cerevisiae* decreased the chlorophyll content as well as shoot system length and number of fibrous roots. On the other hand the addition of *S. cerevisiae* to cultivated soil mixed with potato waste increased plant parameters comparing with the control. Oxidized enzymes (Catalase and Peroxidase) decreased with plant development cultivated in soil amended with potato waste and inoculated with *S. cerevisiae*, while increased in plant cultivated in soil amended with potato waste without inoculated by *S. cerevisiae*.

Key words: *Saccharomyces cerevisia*, Potato wastes, Protease, Biofertilizer.

INTRODUCTION

Wastes from food processing industries represent a severe pollution problem and need better waste management techniques. Increasing world population and the resultant food crisis has shifted emphasis to the availability of 'waste' products of agriculture that could be utilized for alleviating food shortages (Bhattacharjee, 1970).Proteases are important from an industrial perspective due to their wide scale applications in the detergents, food, pharmaceuticals, chemicals,leather, paper and pulp and silk industries (Krik *et al*., 2002; Chellapandi 2010). Owing to the better cleansing properties of protease based detergents and pollution-alleviating capacity over conventional synthetic detergents (Krik *et al*., 2002), alkaline proteases have made their way as key-ingredients in detergent formulations. These proteolytic (protein digesting) biocatalysts have been in use for many centuries, at first in the dairy industry as milk-clotting agents (rennet) for the manufacture of cheese. Proteases are enzymes that catalyse hydrolytic reactions in which protein molecules are degraded to peptides and amino acids. These constitute a very large and complex group of enzymes, which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature optima and stability profile. The specificity of proteolytic enzymes is governed by the nature of the amino acid and other functional groups (aromatic or aliphatic or sulphur-containing) close to the bond being hydrolysed. Proteases are present in all living beings and play an important role in normal and abnormal physiological conditions, catalyzing various metabolic reactions (Sandhya 2004; Sumantha *et al.* 2006).

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Boehm *et al*. (1993); Bowers and Lock (2000); Bardin and Huang (2003) and Bardin *et al*. (2004) found that plant wastes or animal manures contain high values of nitrogenous compounds that could be converted to simple nitrogenous compounds by the effect of enzymes produced by *B. subtilis* when grown within these

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wastes. They also showed that straw fermentation by microbes added, led to the accumulation of ammonia in great amounts by reduction of nitrates present in the straw. Pavalou and Vakalounakis (2005), reported that, these wastes and its ingredients indicate the possible involvement of induced host-plant resistance. Various factors are known to influence fermentation processes. These include carbon and energy source requirements, oxygen demand and supply, temperature, pH, nitrogen, phosphorus and potassium requirement (Pirt, 1975). The decomposition of the wastes may supply the plant with some nutrients particularly microelements present in these wastes (Fuchs and Larbi, 2004).Microorganisms in a soil form part of the biomass and contribute to the reserve of soil nutrients and are generally referred to as the microbial biomass (Insam, 1990, Moreno *et al*. 1999). The application of compost increases the percentages of organic matter,nutrient levels (providing a slow fertilization action over a long period of time), microbial biomass and improves the soils' physical properties (aeration, water holding capacity, etc.) (Bertran, *et al*.2004).

Applying organic materials to crop soil not only generates a better nutritional state, but furthermore, positively influences other properties, such as soil particles aggregation, water holding capacity and aeration (Pagliai *et al.*, 2004, Valarini et al 2009), contributing to generating high production, even with a low or nil application of fertilizers.

MATERIALS AND METHODS

Organism Used:

A commercially bakery yeast (*Saccharomyces cerevisiae*) was obtained from Matroh company of Egyptian Industrial sector. It was cultured on seed media with the composition (g /L): glucose 10. It refreshed for 20 minutes at 30°C in an incubator this served as the starter culture for ethanol production. The base components of culture medium were 10 g Industrial wastes, sterilized at 121 °C for 15 min.

Collection of Samples:

Industrial Potato solid wastes were collected from Assuit Manufactory at Assuit governorates. The samples were placed in a double sterile polyethylene bags (to minimizes the loss of water content and provides sufficient aeration), sealed, transferred immediately to the laboratory, kept in cool place (5ºC) till used in protease production. Chemical analysis of the Potato solid wastes under study (the Mean values of gross%) were: moisture content, 77.0; crude protein, 2.52; crude fat, 0.13; Crude fiber, 3.50; Ash, 5.31; carbohydrate, 88.54). Also the determination of micro and macro-element (mg./ kg^{-1} Dry Mater) were: Fe, 87.75; Mn, 5.25; Cu, 11.45; Zn, 15.9; Na 1350; K, 11002; Ca, 2800; Mg, 1560; S, 2295; P, 2050 (Darwish *et al.*, 2009).

Fermentation Medium:

Fermentation: Liquid nutrient medium containing (g/l) $ZnSO_4 \cdot 7H_2O$ 0.2 and $FeCl_3 \cdot H_2O$ 0.014.Ten grams of solid substrates were, putted in a 250ml Erlenmeyer flask, and mixed with 10 ml of the nutrient medium, sterilized at 121 °C for 15 min, and inoculated with 10% (V/W) of inoculum. The flasks were incubated at 28°C in an incubator under stationary conditions.

Preparation of Crude Enzyme Extract:

Culture media and cells were harvested after 120 h by addition of 100 ml of sterilized distilled water. The mixture was vigorously stirred for about 15 min and filtered through Whatman No. 1 filter paper on Büchner funnel, and centrifuged at 6000 rpm and 4 °C for 15 min. The supernatant thus obtained was used as crude enzyme preparation. While the contents of filter paper used in biofertilizer field.

Preparation of Biofertilizer:

2.6. Determination of Protease Activity:

Protease assay was performed according to Chi *et al*., 2007 with little modifications as follows: The cell culture was centrifuged at 5000 rpm and 4 °C for 10 min. The supernatant (0.5 ml) was mixed with 1.0 ml of 0.5% casein solution in glycine–NaOH buffer (0.05 M, pH 7.5), preincubated at 45 °C for 30 min. The mixture was incubated at 45 °C for 30 min and 2 ml of 10% TCA (trichloroacetic acid) solution was added to the mixture immediately to stop the reaction. The reaction mixture was centrifuged at 10.000 rpm and 4 °C for 10 min. Tyrosine content in the supernatant was determined colorimetrically at 650 nm by using Folin–phenol reagent (Lowry *et al.*, 1951).

The enzyme activity was defined as the amount of the enzyme that liberated 1 µg of tyrosine per minute under the conditions used in this study. The specific protease activity was units per mg of protein. Protein

concentration was measured by the method of Bradford and bovine serum albumin served as standard (Bradford, 1976).

Parameters Controlling Protease Productivity:

Temperature:

The medium (50 g solid potato wastes and a pH 6.0) was inoculated for 72 h with 10% (v/w) yeast culture and incubated at different temperatures (15, 20, 30, 40, 50, and 60 °C).255

Initial pH:

The wastes used were adjusted at different pH values viz. 3, 5, 7, 9 and 11, NaOH-HCl buffer and then inoculated with 10% (v/w) yeast culture and incubated as above.

Carbon Source:

The previous medium was mixed with different nitrogen sources in concentration (0.1%) (Sorbitol, manitol, lactose, fructose, maltose, cellulose, sucrose and starch) and inoculated and incubated at 20°C.

Nitrogen Source:

The previous medium was mixed with different nitrogen sources in concentration (0.1%) (peptone, Ammonium phosphate, Ammonium sulfate, Casein, Glysine and Gelatine) and inoculated and incubated at 20°C.

Incubation Period:

A series of flasks containing 25 g solid potato wastes were incubated at different times (48, 72, 120 and 168 h). Triplicate flasks in each time, were inoculated and incubated aerobically at 20 °C.

Substrate Concentration:

A series of flasks containing different solid potato wastes concentrations (g) 1, 2.5, 5, 10,15, 25 and 50. Triplicate flasks in each concentration, were inoculated and incubated aerobically at 20 °C for 72 h.

Plant Cultivation and Their Treatments Application:

Seeds of *Zea mayes* were cultivated in 15 cm diameter plastic pots containing 1.5 kg autoclaved soil. The cultivated soil was treated as following :(1) amended with 10 g of potato wastes and mixing before autoclaving. (2) amended with *Saccharomyces cerevisiae*. (3) amended with 10 g of potato wasts and mixing before autoclaving and then amended with *S. cerevisiae*. (4) amended with filtrate metabolized medium of *S. cerevisiae* cultivated on potato wastes for 3 days.Control, soil without any treatment. After 25 days of cultivation growth parameters of plant were measured.

Quantitative Determination of Chlorophylls:

Chlorophylls content was determined according to Vernon and Seely (1966) using the following equations:
mg chlorophyll a / gm tissue = 11.63 (A665) -2.39 (A649).
mg Chlorophyll b / gm tissue = 2.11 (A649) -5.18 (A665).
Where (A), denotes the reading of the optical density

RESULTS AND DISCUSSION

The determination of optimal growth conditions yielding the highest proteases activities was achieved by analyzing the influence of several factors. It is known that temperature is one of the most critical parameters that has to be controlled in bioprocess (Chi and Zhao, 2003). The effect of temperature profiling on protease enzyme activity are shown in Figure (1). The results revealed that the specific protease activity was reached to 255 U/mg protein when the strain was grown at 20 °C. Chi *et al.* (2007), revealed that the specific protease activity reached the highest when the yeast strain was grown at 24.5 °C. *Saccharomyces cerevisiae* could grow and produce extracellular protease over a wide range of pH (3.0–7.0). Maximum protease production was obtained at pH 6.0 (Fig. 2). However, optimum production (195 U/mg protein) was obtained at pH 5 and the highest production (336 U/mg protein) was observed at pH 6.0. Similar result was obtained by Chi, *et al*,(2007) which showed that the yeast strain produced the highest yields of alkaline protease (447.5 U/mg protein) at initial pH 6.0 of the production medium. In contrast Chellapandi (2010) showed pH optimum in the range of

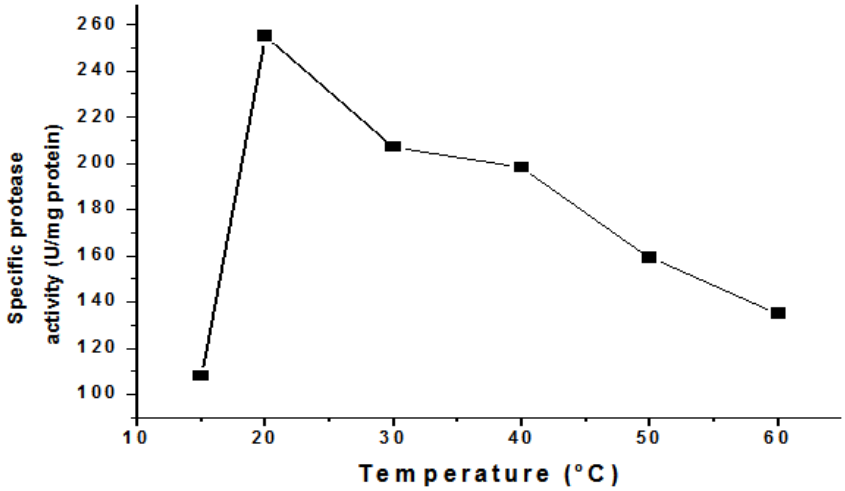

Fig. 1: Effects of different incubation temperatures on protease production.

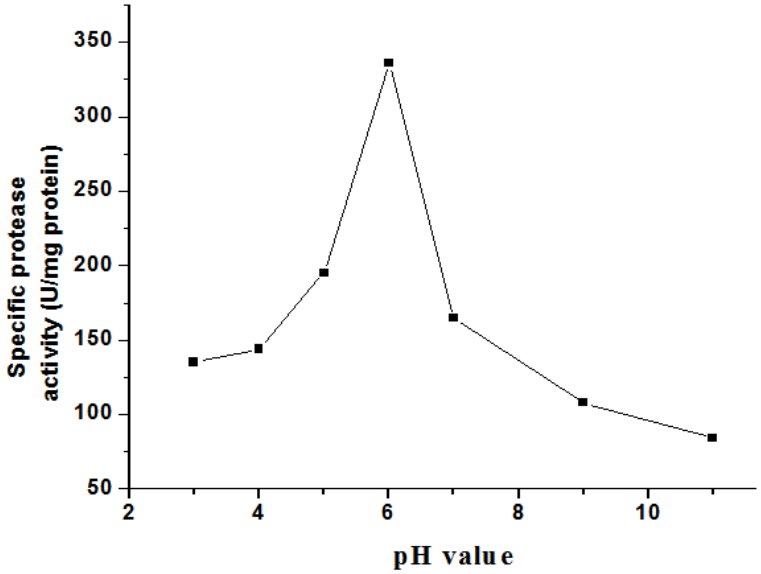

Fig. 2: Effects of different pH values on protease production.

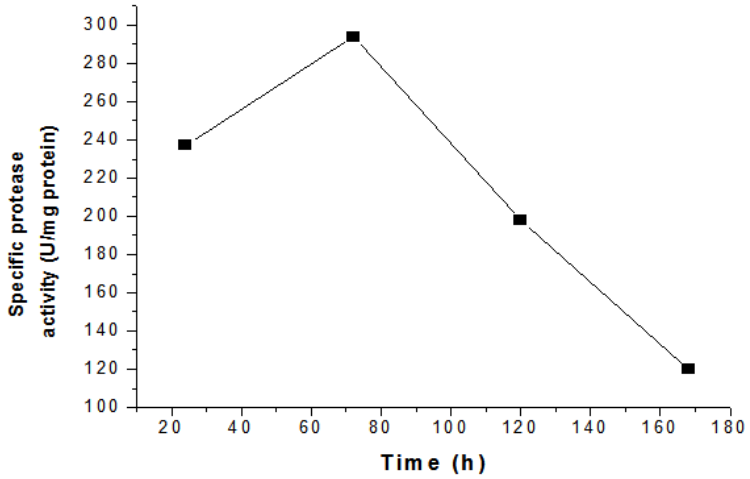

Fig. 3: Effects of different incubation times on protease production.

9-11 of protease production by filamentous fungi. However, for increased protease yields from alkalophilic microorganisms, the pH of the medium must be maintained above 7.5 throughout the fermentation period (Aunstrup, 1980). It has been noted that the important characteristic of most microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production (Kurmar and Tagaki, 1999).

The extracellular proteases activity increased with the increase of fermentation time. The maximum proteases activity (294 U/mg protein) was obtained after 120 h of incubation period. The *Saccharomyces serevisiae* produced a large amount of extracellular proteases activities (237 U/mg protein) detected after 48h and reached the maximum level after 120 h (Fig.3). Under the optimal conditions, 623.1 U/mg protein (7.2 U/ml) of protease activity was reached in the culture of strain *Aureobasidium pullulans* 10 within 30 h of the fermentation when the cell growth reached mid-log phase(Chi *et al*., 2007).

The results in Fig. (4) showed that maltose was the best carbon sources for protease production. The specific protease activity in the culture supernatant was 315 U/mg protein. This meant that strain could secrete extracellular protease and used maltose as sole carbon source for protease production. At the same time our results showed that in the presence of other carbon sources, there was a reduction in protease production. This could be due to catabolite repression by high glucose available in the medium. However, increased yields of alkaline proteases were reported by several other workers who used different sugars such as lactose, sucrose and fructose (Malathis and Chakraborty, 1991; Phadatare *et al*., 1993;Tsuchiya *et al*,1991)

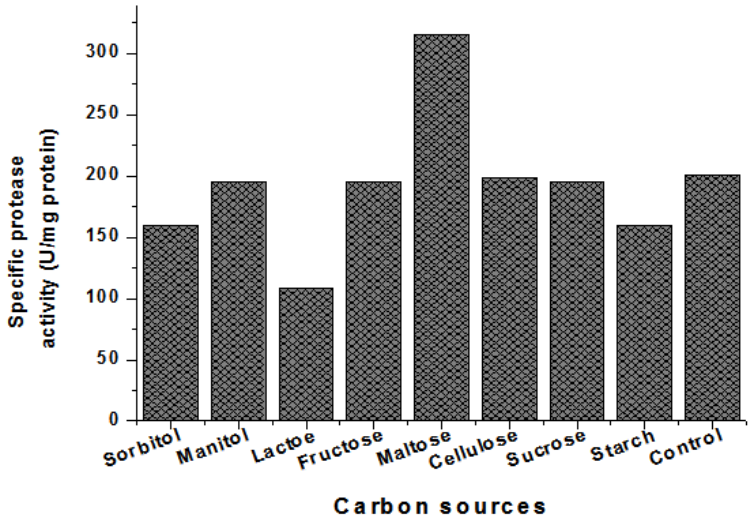

Fig. 4: Effects of different carbon sources on protease production.

It has been reported that effects of a specific nitrogen supplement on protease production differ from organism to organism although complex nitrogen sources are usually used for alkaline protease production (Kurmar and Tagaki, 1999). Fig. (5) showed that ammonium sulfate was stimulatory for protease production by the yeast strain. Specific protease activity in the presence of 1.0% ammonium sulphate reached 240 U/mg protein. The specific protease activity in the supernatant of the yeast strain reached the highest when the production medium contained 1.0% sodium nitrate.

As shown in Fig. (6), the highest protease productivity was achieved at a substrate concentration of 25 g and decreased beyond this concentration. The maximum protease activity (360 U/mg protein) was obtained in culture grown at 15g. The results in Fig. 6 indicated that the optimal concentration of potato waste for the maximum protease production by the yeast strain was 15g. Under this condition, the specific protease activity in the culture reached 360U/mg protein. Upper and under this concentration condition, the specific protease activities were decreased. Chi *et al* (2007) revealed that the optimal concentration of soluble starch for the maximum protease production was 2.5%. Under this condition, the specific protease activity in the culture reached 434 U/mg protein.

Application of biologically treated agro-wastes was able to increase soil microbial activity and plant development, which are highly depressed in many stressed areas. Thus, these treatments can be used as a valuable strategy in desertified areas (Medina *et al* 2004). In our study, the addition of potato waste to cultivated soil without treatment with *Saccharomyces cerevisiae* decreased the chlorophyll content as well as

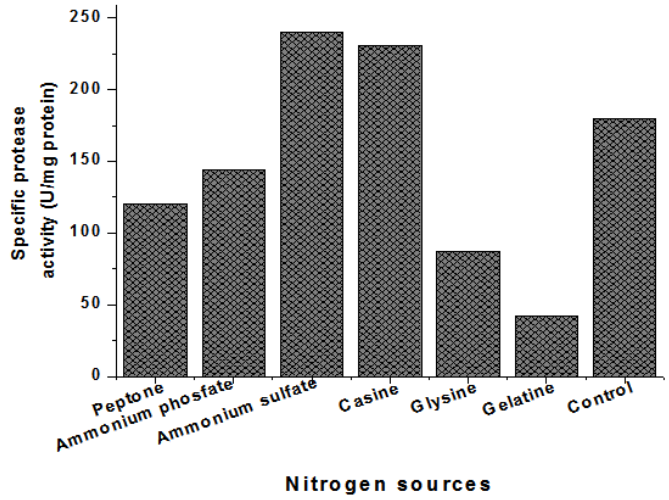

Fig. 5: Effects of different nitrogen sources on protease production.

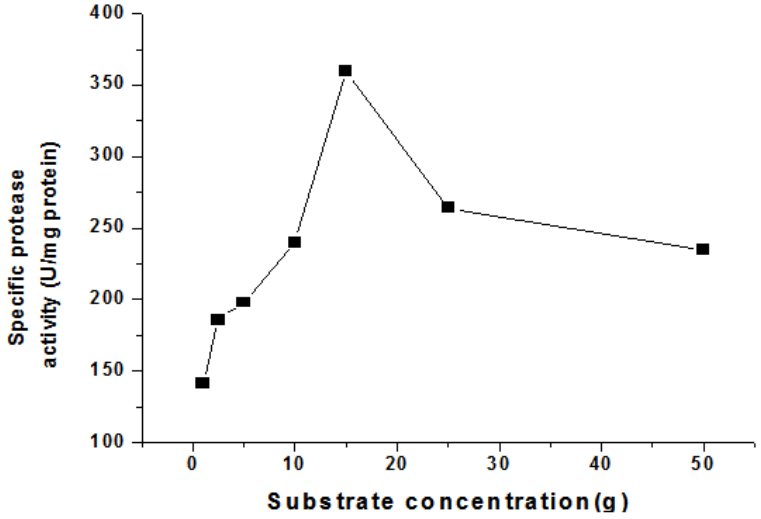

Fig. 6: Effects of different substrate concentrations on protease production.

Shoot system length and number of fibrous roots (tables and Fig. 7).On the other hand the addition of *S. cerevisiae* to cultivated soil mixed with potato waste increased plant parameters including shoot system length and number of fibrous roots and stem width comparing with the control, this may be due to that yeast degradable the complexes compound present in potato wastes which increased soil fertility. Recently Saritha and Maruthi (2010) stated that soil fertility increased with using fungi as degradable agricultural wastes. Surprisingly chlorophyll content and plant growth parameters decreased in soil mixed with metabolized medium metabolized medium of *S. cerevisiae* cultivated on potato waste this may explained as a result of presence of alcoholic compound in metabolized medium which repressed the plant growth, this indicated by the higher amount of oxidized enzymes (Catalase and Peroxidase) in this treatment (Table 2).

Table 1: Growth parameters of *Zea mayes* plant cultivated different treatments.

Growth parameter	*Zea mayes* cultivated in				
	Soil	Soil + P	Soil +P +S	Soil+ S	Soil + Mm
Shoot length (cm)	23	20	25	24	19
Stem width (cm)	0.8	0.7	1.1	0.9	0.5
Fibrous Root number	10	8	12	12	8

P, potato waste; S, *Saccharomyces cerevisiae;* Mm, metabolized medium

Table 2: Chlorophyll content and Enzymes of *Zea mayes* at different treatments

Chlorophyll and Enzyme type	Chlorophyll and Enzyme of *Zea mayes* cultivated in				
	Soil	Soil + P	Soil +P +S	Soil+ S	Soil + Mm
Chlorophyll (a)	6.45	4.42	8.34	6.88	4.22
Chlorophyll (b)	2.32	2.27	2.98	2.05	1.62
Catalase	25.55	31.56	24.23	26.45	35. 43
Peroxidase	0.066	0.081	0.079	0.053	0.082

P, potato waste; S, *Saccharomyces cerevisiae;* Mm, metabolized medium

Fig. 7: Growth parameters of *Zea mayes* plant cultivated different treatments A, control soil without any treatment; B, soil mixed with potato waste; C, soil mixed with potato waste and inoculated with *S. cerevisiae*.; D, soil inoculated with *S. cerevisiae*.; E, soil mixed with metabolized medium of *S. cerevisiae* grown on potato waste.

REFERENCES

Aunstrup, K., 1980. Protenases. In: A.H. Rose, Editor, *Economic Microbiology: Microbial Enzymes and Bioconversions*, Academic Press, New York, 50-114.

Bardin, S.D. and H.C. Huang, 2003. Efficacy of stickers for seed treatment with organic matter or microbial agents for the control of damping off sugar beet. Plant Pathol. Bull, 12: 19-26.

Bardin, S.D., H.C. Huang and J.R. Mayer, 2004. Control of Pythium damping – off of sugar beet by seed treatment with crop straw powder and a biocontrol agent. Biological control, 29: 452-460.

Bertran, E., X. Sort, M. Soliva and I. Trillas, 2004. Composting Winery Waste: Sludges and Grape Stalks, Bioresource Technol, 95: 203-208.

Bhattacharjee, J.K., 1970. Microorganisms as potential sources of food, Adv. Appl. Microbiol., 13: 139.

Boehm, M.J., L.V. Madden and H.A.J. Hoitink, 1993. Effect of organic matter decomposition level on bacterial species diversity and composition in relationship to *Pythium* damping-off severity. Appl. Environ. Microbiol., 59: 4171-4179.

Bowers, J.H. and J.C. Locke, 2000. Effect of botanical extracts on the population density of *Fusarium oxysporum* in soil and control of fusarium wilt in greenhouse. Plant Dis., 84: 300-305.

Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem., 72: 248-253.

Chellapandi, P., 2010. Production and Preliminary Characterization of Alkaline Protease from *Aspergillus flavus* and *Aspergillus terreus* E-Journal of Chemistry, 7(2): 479-482.

Chi, Z. and S. Zhao, 2003. Optimization of medium and cultivation conditions for pullulan production by a new pullulan-producing yeast, Enzyme Microb. Technol., 33(2003): 206-221.

Chi, Z.C., Ma, P. Wang and H.F. Li, 2007. Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. Bioresource Technology, 98(3): 534-538.

Darwish, S.M.I., M.M. Afifi, E.M. Mostafa and A.A. El-Shanawany, 2009. Production of Amylase Enzymes by Filamentous Fungi. Assuit Univ. J. Botany, 38(2): 1-14.

Fuchs, J.G. and M. Larbi, 2004. Disease control with quality compost on pot and field trials 1st International Conference Soil and Compost Eco-Biology September 15 th – 17th; Leon – Spain.

Insam, H., 1990. Are the Soil Microbial Biomass and Basal Respiration Governed by Climate Regime?, Soil Biol Biochem., 22: 525-532.

Kirk, O., T.V. Borchert and C.C. Fuglsang, 2002. Industrial enzymes applications. Curr. Opin. Biotechnol., 13: 345-351.

Kurmar, C.G. and H. Tagaki, 1999. Microbial alkaline protease: from bioindustrial viewpoint, Biotechnol. Adv., 17: 561-594.

Lowry, O.H., N. Rosebrough, A. Farr and R. Randall, 1951. Protein measurement with Folin phenol reagent, J. Biol. Chem., 193: 265-275.

Malathis, S. and R. Chakraborty, 1991. Production of alkaline protease by a new *Aspergillus flavus* isolate under solid substrate fermentation conditions for use as a depilation agent, Appl. Environ. Microbiol., 57(1991): 712-716.

Medina, A., N. Vassilev, M.M. Alguacil, A. Roldán, R. Azcón, 2004. Increased Plant Growth, Nutrient Uptake, and Soil Enzymatic Activities in A Desertified Mediterranean Soil Amended With Treated Residues and Inoculated With Native Mycorrhizal Fungi and A Plant Growth-Promoting Yeast. Soil Science, 169(4): 260-270.

Moreno, J.L., T. Hernández and C. Garcia, 1999. Effects of a Cadmium-contaminated Sewage Sludge Compost on Dynamics of Organic Matter and Microbial Activity in an Arid Soil, Biol Fertil Soils, 28: 230-237.

Pagliai, M., N. Vignozzi and S. Pellegrini, 2004. Soil structure and the effect of management practices. Soil Tillage Res., 79: 131-143.

Pavlou, G.C. and D.J. Vakalounakis, 2005. Biological control of root and stem rot of greenhouse cucumber caused by *Fusarium oxysporum* f. sp. *Radicis cucumerinum* by lettuce soil amendment. Crop Protec., 24: 135-140.

Phadatare, S.U., V.V. Deshpande and M.C. Srinirasan, 1993. High activity alkaline protease from Conidiobolus coronatus (NCL 86.8.20): enzyme production and compatibility with commercial detergents, Enzyme Microb. Technol., 15: 72-76.

Pirt, S.J., 1975. Principles of Microbe and Cell Cultivation. Blackwell Scientific Publications, Oxford.

Sandhya, C., A. Sumantha, A. Pandey, 2004. Proteases. In: Enzyme Technology, A. Pandey, C. Webb, C.R. Soccol, C. Larroche (Eds.), Asiatech Publishers Inc., New Delhi, India pp: 312-325.

Saritha, V. and Y.A. Maruthi, 2010. Soil fungi : Potential mycoremadiator of lignocelluloic waste. Bioresources, 5(2): 920-927.

Sumantha, A., L. Christian and P. Ashok, 2006. Microbiology and Industrial Biotechnology of Food-Grade Proteases: A Perspective Food-Grade Proteases, Food Technol. Biotechnol., 44(2): 211-220.

Tsuchiya, K., H. Sakashita, Y. Nakamura and T. Kimura, 1991. Production of thermostable alkaline protease by alkalophilic Thermoactinomyces sp. HS682, Agric. Biol. Chem., 55: 3125-3127.

Valarini1, P., C. Gustavo, S. Alex, M. Karina, R. Rosa, C. Pablo and B. Fernando, 2009. Effect of compost application on some properties of a volcanic soil from central south chile Chilean. Journal of agricultural research, 69(3): 416-425.

Vernon, L.P. and G.R. Seely, 1966. The chlorophylls. Academic Press, New York, London.