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Enhancement Of Actinobacterial Protease Production by Optimizing Fermentation Parameters

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Abstract

Actinomycetes were isolated from soil, compost pit and vermicompost samples. Protease producing actinomycetes were screened and out of twenty five cultures,ten were positive for protease production. Actinomycete isolate MG4 was selected for optimizing submerged fermentation technology. MG4 produced 264EU/ml of protease in submerged fermentation.The selected isolate grew on Bennett's medium with typical powdery texture and ivory aerial spore mass. MG4 exhibited straight chain spore arrangement pattern on morphological analysis by slide culture technique.Optimization of protease production process was done and the maximum production was observed at 30°C and pH 7 in72 hours. Medium containing soybean residue promoted the protease production to maximum (316 EU/ml).

Introduction

Actinobacteria are filamentous prokaryotes, rich in GC content. They are a peculiar group of bacteria predominantly present in soil. They are slow growers and play a very important role in mineralization. Actinobacteria can breakdown a wide variety of organic macromolecules which are difficult to degrade. The extensive range of enzymes produced by them allow these filamentous bacteria to survive in extreme climates also. Actinomycetes have been isolated from soil [Zhu et al. 2007, Atalan et al. 2000], water [Zaitlin et al. 2003], insects, salt lakes [Thumar and Singh 2007], hot springs [Song et al. 2009], marine environments etc [Tian et al. 2009]. Production of protease

[Subramaniet al. 2009], amylase, cellulase, glucose isomerase etc[Bhasin and Modi 2013, Bhasin and Modi 2012] have been reported by numerous researchers. They are also well known producers of antibiotics[Chater et al. 2006]. Majority of the antibiotics available in the market are produced by Actinomycetes, especially Streptomycetes. They have extraordinarily large genome size which accounts for their special capacity to produce a vast repertoire of metabolites and enzymes.Enormous capacity of extracellular enzyme production exhibited by Actinomycetes help in recycling of organic and inorganic matter in the ecosystem [Vonothini et al. 2008, Bascarn et al. 1990].

Present industrial global market for enzymes is more than $ 2 billion which is expected to rise even further [Thumar et al. 2007].Proteases find huge application in industrial market and accounts for 25% of total enzyme sales.

Majority of the proteases produced industrially is of microbial origin [Mehta et al. 2006].Proteases are the degradative enzymes, besides catalyzing the hydrolysis of proteins they are also involved in specific modifications of proteins such as activation of zymogens [Rao et al. 1998].Variety of proteases are produced using Actinomycetes for industrial applications. Submerged as well as solid state fermentation process are employed for the production of protease using Streptomycetes [Yang and Wang 1999, De Azeredo et al. 2006]. Mitra and Chakraborty (2005) have reported the presence of multiple kinds of proteases in the fermented broth of actinomycetes. Proteases find application in laundry, food, medicine, cosmetic, pharmaceutical industry and other biotechnological purposes [Ellaiah et al. 2002].Numerous studies are associated with halophilic and alkalophilic proteases of bacterial origin [Thumar et al. 2007].

Plant and animal sources are also used for the production of proteases but microbial proteases are preferred because of low cost of production, wide pH and temperature range of enzyme activity and stability. They are also preferred for large scale production due to the ease of growth and production on economic grounds also.Microbial proteases are extracellular in majority of the cases making the production and recovery process convenient. Numerous bacteria such as *B. cereus, B. licheniformis, B. mojavensis, B. megaterium and B. subtilis, Streptomyces clavuliyerus, Streptomyces nogalator, Streptomyces fungicidicus[Subramani* et al. 2009, Mitra and Chakraborty 2005, Bascarn et al. 1990] and fungi for example *Aspergillus flavus, Aspergillus melleu, Aspergillus niger, Chrysosporium keratinophilum,* Fusarium graminarum, Penicillium griseofulvis, Scedosporium apiosermumare known to produce proteases[Nijland and Kuipers2008, Rao et al. 1998].Microbial protease production technology can be further improved by developing recombinant enzymes [Jisha et al. 2013]. The influence of climate on plants and quality of food and their scarcity in case of animals is a major hurdle for these sources of proteases. Fermentation process parameter optimization and use of economic agricultural residues make the microbial production technology highly acceptable.

Current investigation deals with isolation and screening of protease producing actinomycetes. Screening was done at qualitative as well as quantitative level. High yielding isolate was studied for optimization of submerged fermentation technology wherein medium combination, fermentation period, optimum pH and temperature were determined.

Materials and Methods

Isolation Of Actinomycetes

Soil samples were collected from garden area, open fields, compost pits and vermicompost. The samples were treated with calcium carbonate and sundried. Suspension of samples were inoculated on plates and incubated at 30°C for 7 days. Actinomycetes were isolated and preserved on Actinomycete isolation agar and Bennett's agar medium. Morphological analysis of the selected isolates was performed by slide culture technique.

Screening For Protease Producers

Primary screening for qualitative protease producers was done on Bennett's agar medium containing milk (5%), casein(3%) and gelatin(1%) separately. All the isolates were spot inoculated on medium containing protein substrates and incubated at 30°C. Zone of hydrolysis was observed on milk Bennett's agar plates by the difference in transparency developed in the medium. Casein Bennett's agar was observed for visualization of hydrolysis by development of a clear area, a halo around the isolate's growth. Commassie brilliant blue was used for visualization of the zone of hydrolysis on gelatin Bennett's agar plates [Vermelho et al. 1996].

Submerged Fermentative Production For Secondary Screening

Secondary screening for selected isolates spotted by primary screening was performed by submerged fermentation process. Comparison of protease production was done performing submerged fermentation process in Bennett's medium. The isolates were inoculated in 100ml conical flasks containing 20 ml of Bennett's medium. The flasks were incubated at 30°C and 100 RPM in orbital shaker [Remi 24CL] for 96 hours [Mehta et al. 2006].

Preparation Of Crude Protease Extract

The fermentation process was terminated on fourth day of incubation and the fermented broth was harvested in sterile centrifuge tubes. Crude protease extract was prepared by centrifugation of fermented broth at 5000 RPM for 10 minutes.

Determination Of Protease Activity

Protease production by different actinomycete isolates was determined by Nagase's method. Protease assay is a modification of two methods viz. Hagihara, 1953 and Anson, 1938. Casein was used as the substrate and protease activity was determined by estimating the soluble tyrosine released. Tyrosine was determined spectrophotametrically using Folin reagent. To 5ml of purified casein solution 1ml of crude enzyme extract (1:1 diluted with acetate buffer) was added. This was incubated for 10 minutes at 30°C. The reaction was terminated by adding 5ml of tri-chloro acetic acid. This was incubated at 30°C for 30 minutes for precipitating remaining total protein. The precipitates were separated by centrifugation. 2 ml of supernatant was mixed with 5 ml of sodium carbonate and 1ml of 1N Folin reagent. This was incubated for 30 minutes at 30°C. The absorbance for determination of tyrosine value was measured at 660 nm and expressed in Proteolytic Unit of Nagase (PUN). One PUN is defined as the amount enzyme which acts on casein for 10 minutes at 30°C and produces a quantity of Folin color-producing substances not precipitated by trichloroacetic acid that is equivalent to 1µg of tyrosine.

Optimization Of Protease Production

Demand of microbial enzymes is increasing tremendously because of varied applications [Jisha etal.2013]. Continuous research is going on for establishing an effective production technology

which is economically viable. Production of protease was optimized by determining the optimum temperature and pH,fermentation period and medium ingredients required for maximum production.

Determination Of Optimum Temperature For Protease Production

All the microbes require specific temperature for the production of enzymes and metabolites. The actinomycete isolate MG4 was subjected to submerged fermentation process in Bennett's broth. Protease production was studied at 15°C, 20°C, 25°C, 30°C, 35°C, 45°C & 50°C. Protease produced at different incubation temperatures was estimated by Nagase's method

Optimum P^H For Protease Production

Effect of pH on production of protease was studied by growing the isolate in Bennett's broth. Submerged fermentation was carried out for the actinomycete isolate MG4 with initial pH adjusted to 5, 6, 7, 8, 9, 10 and 11. Optimum pH was determined by estimating the enzyme produced using Nagase's method.

Determination Of Optimum Fermentation Period For Protease Production

Optimum fermentation period was determined by performing submerged fermentation process for the actinomycete isolate MG4 for different time intervals. Seven flasks containing Bennett's medium were inoculated with actinomycete isolate MG4 and incubated for protease production at 30°C and 100 RPM. Fermentation process was terminated in one conical flask after a fixed interval of 24 hours for continuously seven days. The extent of protease produced was determined by preparation of crude enzyme extract and determining the activity by Nagase's method.

Selection Of Suitable Production Medium For Protease Production

Production of enzymes significantly depends on the medium composition and different organisms are influenced by different medium components. The selected isolate was subjected to submerged fermentation in six different media to obtain maximum protease production. Media used by various researchers were inoculated with the selected actinomycete isolate such as Medium I consisting of Casein, 2%; Glucose, 0.1%; KH_2PO_4, 0.15%; proposed by Chahal et.al. (1976). Kathiresan (2007) reported the production of protease in a medium containing Casein, 5%; Glucose, 5%; Peptone, 5%, Yeast Extract 5%,; $MgSO_4$,0.1%, K_2HPO_4, 0.25%, $FeSO_4$, 0.1%, was used as Medium No. II. Media No. III was proposed by Shirato (1965) which contains Glucose, 3.5gm%; Defatted soybean, 2.5%; Dried beer yeast, 0.3%; Ammonium sulphate, 0.2%; Calcium carbonate, 0.2%; Sodium chloride, 0.2%; Soybean oil, 0.24%.

Media No. IV contains Starch, 1.5%; Milk, 1.5%; K_2HPO_4, 0.3%; Yeast extract, 0.1%; $MgSO_4$, 0.05%; $NaHCO_3$, 1% and Medium No. V contains Gelatin, 1%; Peptone, 0.5%; Yeast extract, 0.5%, NaCl, 5%; proposed by Thumar et. al. (2007). Bennett's broth medium was also used for reference and named as Medium No. VI. The actinomycete isolate MG4 was inoculated in 20ml of above media in 100ml conical flasks and fermentation process was carried out for 96 hours at 30°C and 100 RPM. Crude enzyme extract was prepared by centrifuging the fermented broth at 5000 RPM for 10 minutes at 4°C. The comparison of protease production was done by determining enzyme activity by Nagase's method.

Results and Discussion

Microorganism

Actinomycetes were isolated and screened for protease production. Culturally and morphologically diverse twenty five actinomycete cultures were isolated from compost and soil samples. The samples were rich in actinomycetal cultures. They grew on Bennett's agar and Actinomycete isolation agar medium with their typical earthy smell of geosmin.Geosmin is an organic compound which has the specific earthy flavor and is produced by actinobacteria and some other soil dwelling microorganisms [Juttner and Watson, 2007].Actinobacteria possess tremendous adaptability to diverse environmental conditions. They have been isolated from extremely cold and hot environments [Cotarlet 2009, Song et. al. 2009]. Our collection of isolates consisted of 40% grey aerial spore mass bearing cultures and another 48% white, rest of them exhibited ivory and green aerial spore mass (fig.1). Pigmentation which is a common feature with actinomycetes was observed in 40% of our isolates. Slide culture technique revealed the spiral spore arrangement pattern of grey aerial spore mass bearing isolates.Straight chain spore patterns were observed with white and ivory colour spore mass bearing isolates. The isolate selected for this study exhibited ivory spore mass colour and straight chain spore pattern arrangement, according to Bergey's Manual of Systemic Bacteriology Volume 4 *Kitasatosporia* exhibits such features.

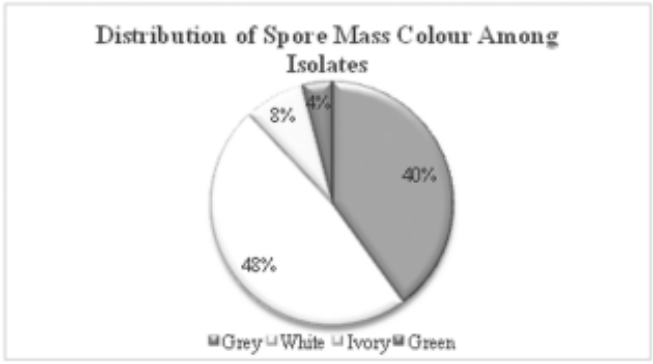

Fig 1.Distribution of Spore Mass Colour among Isolates

Qualitative And Quantitative Screening For Protease Producing Actinomycete

Ten isolates were found to be positive for protease production out of which five were high yielding. The isolates were analyzed for degradation of casein and gelatin along with hydrolysis on milk agar plates. Large hydrolysis zones ranging between 24 to 44mm were produced by promising isolates (fig.2). Actinomycete isolate MG4 was selected for further studies as it exhibited highest productivity of 264 EU/ml of the fermented broth in submerged fermentation (Fig.3).Protease production strategy was optimized using the selected isolate MG4.

Protease production has been reported by numerous strains of actinomycetes[Subramani et al. 2009, Thumar 2007, Rifaat 2006] and Bacillus[Darani 2008]. They are also employed at industrial scale for large scale production. Actinomycetes produce large number of extracellular enzymes in order to survive in different type of environmental conditions with varying organic and inorganic content. They degrade the polymeric macromolecules for their nutritional purpose along with maintenance of ecological balance [Mitra and Chakraborty 2005,Bascarn et al. 1990].

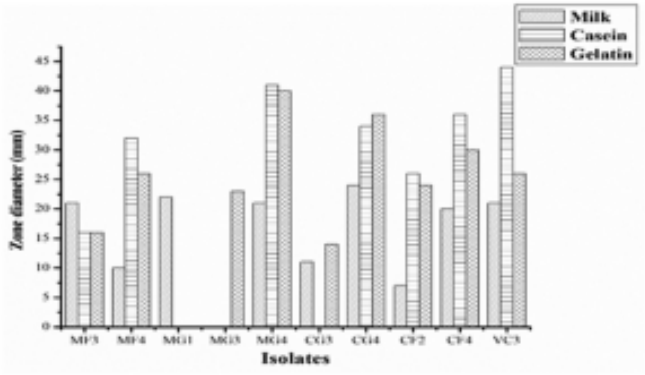

Fig 2. Zone of hydrolysis exhibited by isolates on milk agar, casein agar and gelatin agar plates

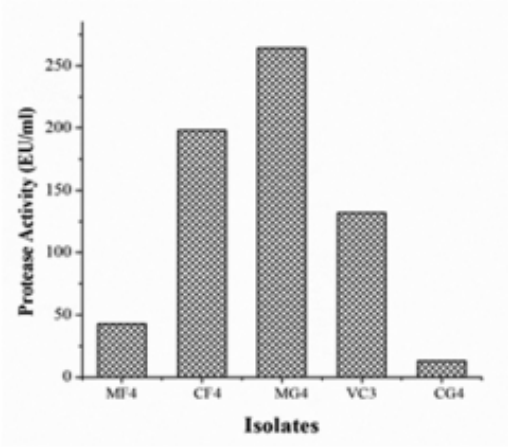

Fig 3. Submerged fermentative production of protease by selected isolates

Optimization Of Submerged Fermentation Process For Protease Production By Actinomycete Isolate MG4

Influential parameters for protease production were studied in order to meet high yield demands for commercialization of production technology.

Influence Of Temperature On Protease Production

Growth and production of protease was maximum at mesophilic range of temperature. Highest protease production was observed at 30°C (Fig.4). Proteases working at mesophilic temperature find applications in degradation of environmental wastes as compared to thermophilic proteases for industrial applications [Sepahy and Jabalameli 2011, De Azeredo 2006]. Kathiresan and Manivannan 2007 also found high protease activity at 30°C. Numerous bacterial proteases produced from Bacillus species have mesophilic and others have thermophillic optimum temperature such as Sepahy (2011) reported 60°C as optimum.

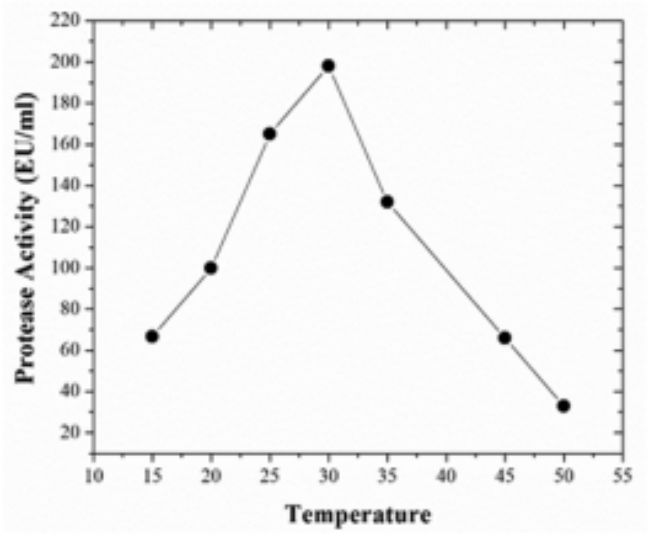

Fig 4. Effect of temperature on protease production

Influence Of P^H On Protease Production

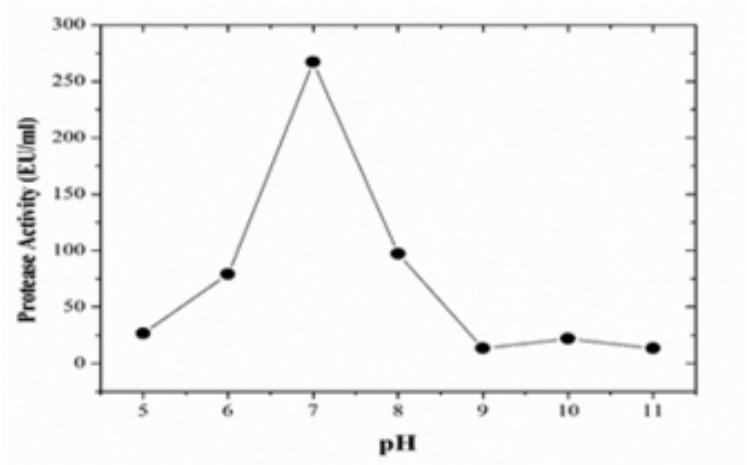

Fig 5. Effect of pH on protease production

Profound effect of pH on protease production and growth of the isolate was observed. The isolate MG4 exhibited very scanty growth and protease production at pH 5 and 6 (Fig.5). Growth and production was also less above pH 9. Highest protease production was observed at pH 7.Components of the medium also alters the pH which in turn influences the protease production. When ammonium salts are used as nitrogen source in the medium, the pH tends to become acidic on its degradation whereas organic nitrogen sources such as amino acids and peptides when utilized by microbes in the production medium leads to alkaline conditions[Ellaiah et al. 2002]. Majority of the reports focus on alkalophillic proteases because of industrial applications [Mehta et al.

2006].Certain applications such as preparation of soluble flavoured protein hydrolysates at industrial level requires proteases with neutral optimum pH range [Rao et al. 1998]. Many proteases for example keratinases are required for degradation of feather wastes at neutral pH.

Determination Of Optimum Fermentation Period

Our study revealed that maximum protease production can be obtained after 72 hours of fermentation process (Fig.6).It is economically favorable that production reaches to its highest in 72 hours, which means that the fermentation process need not to be carried out long to get higher yields as in case of other actinomycete fermentation processes.In some cases Streptomyces are reported to produce protease in 144 hours [Subramani et al. 2009, Kathiresan and Manivannan2007].Fungal proteases also require longer incubation period such as seven days for high yield [Muthulakshmi et al. 2011].Protease is required by the organism for growth starting from the initial stages where the proteinases digests the complex protein macromolecules into peptides. These peptides are further degraded by peptidases to release amino acids for vital activities of the cell. As the growth proceeds, proteases degrade majority of the proteins in the medium including other enzymes.

This leads to high titre of proteases and lesser amount of other enzymes and metabolites in the medium towards stationary phase in some cases. However addition of complex organic protein substrates induces protease production in early stages of growth also.

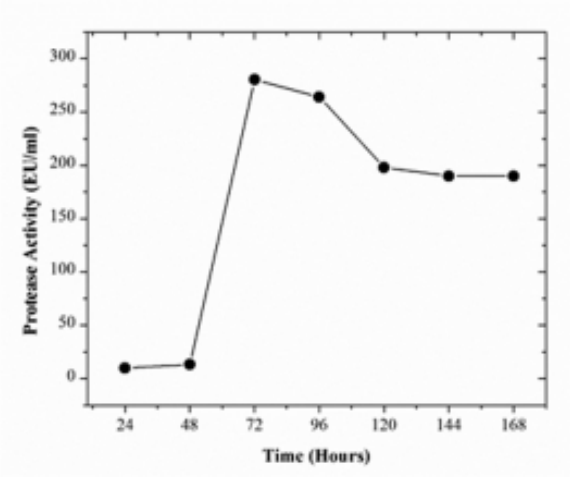

Fig 6. Determination of optimum fermentation period for protease production

Selection Of Suitable Production Medium

All the organisms vary in their growth pattern and production of metabolites, therefore there is no perfectly defined medium available so far which can be used for any organism in consideration [Jisha et, al. 2013]. Production of extracellular protease is highly influenced by medium components [Mehta et al. 2006]. Protease is reported to be inducible in some cases, therefore a variety of medium combinations were studied for protease production. Extent of protease production by high yielding isolate MG4 was compared in presence of casein, gelatin as pure protein containing medium and defatted soybean and milk as crude protein source. A highly encouraging result was obtained, protease was produced in very high amount 316 EU/ml in the medium (Medium No. III) containing

crude protein source (defatted soybean) (Fig.7). Second highest productivity was observed in Bennett's broth (Medium No. VI). Medium containing casein along with yeast extract was next to Medium No. III and VI. Protease production seems to be enhanced in presence of yeast extract which is a common factor in all the three media showing high yield. Although specific organisms require specific nitrogen and carbon sources for optimum production, Ellaiahet, al. (2002) reported a positive impact in most of the cases where complex organic nitrogen sources were used in the medium instead of simpler inorganic sources. However gelatin was not found to be suitable component for protease production in our study.

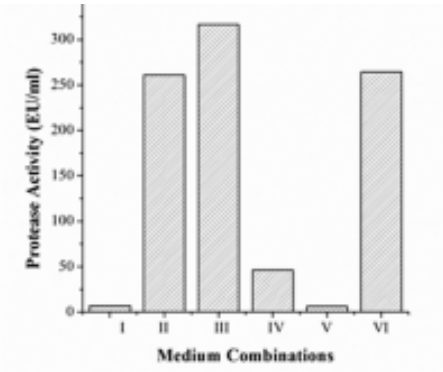

Fig 7. Determination of suitable fermentation medium for protease production

Conclusion

Our study reveals the presence of diversity in actinomycetes present in soil and their extensive capacity to produce proteases. Actinomycetes are already explored for the production of numerous bioactive compounds but still there is tremendous scope available for searching industrially and environmentally useful cultures.The selected isolate exhibited ivory aerial spore mass colour with straight chains of spore pattern arrangement. The isolated culture demonstrated high protease production capacity in Bennett's broth. Growth and production of protease in case of our isolate exhibited synchronous trend, the suitable temperature and pH coincided for both. The optimum pH and temperature for production was found to be 7 and 30°C respectively. Requirement of moderate temperature and neutral pH for growth and production process are definitely advantageous results which opens a way for the application of the enzyme in food industry and environmental purposes. Early production of enzyme, i.e. 72 hours of optimum fermentation period also favors commercialization of the technology. Another encouraging result was the increased protease production in presence of crude nutritive sources.The productivity can further be increased by understanding and modulating the protease synthesis at genetic level.

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References

Anson M. L. (1938)The estimation of pepsin, papain and cathepsin with haemoglobin, Journal of

general physiology, Vol.22: 79-89.

Atalan E., Manfio G.P., Ward A.C., Kroppenstedt R.M. and Goodfellow M. (2000) Biosystematic studies on novel Streptomycetes from soil, Antonie van Leeuwenhoek, Vol. 77 (4): 337–353.

Bascarn V., Hardisson C. and BrafiaA.F. (1990)Regulation of extracellular protease production in *Streptomyces clavuliyerus,* Applied Microbiology and Biotechnology, Vol. 34 (2): 208-213.

Bhasin S., and Modi H.A. (2012) Optimization of Fermentation Medium for the Production of Glucose Isomerase using *Streptomyces* sp. SB – P1, Biotechnology Research International, Vol. 2012, doi:10.1155/2012/874152.

Bhasin S.,and Modi H.A., (2013) Streptomycetes: A Storehouse of Bioactive Compounds and Enzymes; A: Production of Glucose Isomerase, Research Journal of Recent Sciences, 2013,Vol. 2(1):330-339.

Chahal, D.S.and Nanda, S.K.(1976). A screening of some actinomycetes for protease production,Proceedings of the Indian Notational Science AcademyVol. 41(5) 427-434.

Chater K.F.(2006)Streptomyces Inside-Out: A new perspective on the bacteria that provide us with antibiotics, Philosophical Transactions of the Royal Society B Biological Sciences, Vol. 361 (1469): 761-768.

Cotarlet M., Negoita T., Bahrim G. and Stougaard P.(2009)Cold adapted amylase and protease from new Streptomyces antarctic strain, Innovative Romanian Food Biotechnology, Vol. 5: 23-30.

Darani K.K., Falahatpishe H.R. and Jalali M. (2008) Alkaline protease production on date waste by an alkalophilic Bacillus sp. 2-5 isolated from soil, African Journal of Biotechnology, Vol. 7 (10): 1536-1542.

De Azeredo L.A.I., De Lima M.B., Coelho R.R.R. and Freire D.M.G. (2006) Thermophilic protease production by Streptomyces sp. 594 in submerged and solid-state fermentations using feather meal, Journal of Applied Microbiology, Vol. 100 (4): 641–647.

Ellaiah P., Srinivasulu B. and Adinarayana K. (2002) A Review on Microbial Alkaline Proteases, Journal of Scientific& Industrial Research Vol. 61: 690-704.

Hagihara B. (1953)Standard Methods of Biochemical Experiments,Bunkodo, Tokyo, pp 207.

Jisha V.N., Smitha R.B., Pradeep S., Sreedevi S., Unni K.N., Sajith S., Priji P., Josh M.S. and Benjamin S. (2013)Versatility of microbial proteases. Advances in Enzyme Research Vol.1 (3), 39-51 http://Dx.Doi.Org/10.4236/Aer.2013.13005.

Juttner F. and Watson S.B. (2007) Biochemical and ecological control of geosmin and 2-methylisoborneol in source waters, Applied and Environmental Microbiology, Vol. 73 (14): 4395-4406.

Kathiresan K. andManivannanS. (2007)Production of alkaline protease by Streptomyces sp. isolated from coastal mangrove sediment, Research Journal of Environmental Sciences, Vol.1 (4): 173-178.

Mehta V.J., Thumar J.T. and Singh S.P. (2006) Production of alkaline protease from an alkaliphilic actinomycete. Bioresource Technology Vol. 97: 1650–1654.

Mitra P. and Chakraborty P.K. (2005)An extracellular protease with depilation activity from

Streptomyces nogalator, Journal of Scientific and Industrial Research, Vol. 74: 978-983.

Muthulakshmi C., Gomathi D., Kumar D. G., Ravikumar G., Kalaiselvi M. and Uma C. (2011) Production, Purification and Characterization of Protease by *Aspergillus flavus* under Solid State Fermentation Jordan Journal of Biological Sciences,Vol. 4(3): 137 - 148

Nagase and Co., Ltd .Quantitative assay of protease activity. ItachiboriMinamidori 1 –chome, Osaka , Japan.

Nijland R. and Kuipers O.P. (2008) Optimization of protein secretion by Bacillus subtilis, Recent Pat. Biotechnol. Vol. 2: 79–87.

Rao M. B., Tanksale A. M., Ghatge M. S., and Deshpande V. V. (1998) Molecular and Biotechnological Aspects of Microbial Proteases, Microbiology And Molecular Biology Reviews, Vol. 62(3): 597–635.

Rifaat H.M., El-Said O.H., Hassanein S.M. and Selim M.S.M. (2006) Protease activity of some mesophilic Streptomycetes isolated from Egyptian habitats, Journal of Culture Collections, Vol. 5 (1): 16-24.

Sepahy A. A. and Jabalameli L. (2011) Effect of Culture Conditions on the Production of an Extracellular Protease by Bacillus sp. isolated from Soil Sample of Lavizan Jungle Park, Enzyme Research Vol. 2011, Article ID 219628, doi:10.4061/2011/219628.

Shirato S. and Nagatsu C. (1965) Carbohydrate sources for the production of protease and streptomycin. Applied Microbiology, Vol. 13:669-672.

Song Z., Zhi X., Li W., Jiang H., Zhang C. and Dong H. (2009) Actinobacterial Diversity in Hot Springs in Tengchong (China), Kamchatka (Russia), and Nevada (USA), Geomicrobiology Journal,Volume 26(4): 256-263.

Subramani R., Mahalingam R., Narayanasamy M. (2009)Characterization of a thermostable alkaline protease produced by marine *Streptomyces fungicidicus* MML1614, Bioprocess and Biosystems Engineering,Vol. 32(6): 791–800.

Thumar J.T. and Singh S.P. (2007) Secretion of an alkaline protease from a salt-tolerant and alkaliphilic, *Streptomyces clavuligerus* strain MIT-1, Brazilian Journal of Microbiology, Vol. 38 (4):766-772.

Tian X., Tang S., Dong J., Zhang Y., Xu L., Zhang S. and Li W. (2009) Marinactinosporathermotolerans gen. nov., a marine actinomycete isolated from a sediment in the northern South China Sea. International Journal of Systematic and Evolutionary Microbiology Vol. 59: 948–952 doi 10.1099/ ijs.0.005231-0.

Vermelho A. B., Meirelles M. N. L., Lopes A., Petinate S. D. G., Chaia A. A. and Branquinha M. H. (1996) Detection of extracellular proteases from microorganisms on agar plates, Memorias do Instituto Oswaldo Cruz, Rio de Janeiro, Vol. 91(6): 755-760.

Vonothini G., Murugan M., Sivakumar K. and SudhaS. (2008) Optimization of protease production by an actinomycete strain, PS-18A isolated from an estuarine shrimp pond, African Journal of Biotechnology, Vol. 7 (18): 3225-3230.

Yang S.S. and Wang J.Y. (1999) Protease and amylase production of Streptomyces rimosus in

submerged and solid state cultivations, Botanical Bulletin of Academia Sinica, Vol. 40 (4): 259-265.

Zaitlin B., Watson S.B., Ridal J., Satchwill T. and Parkinson D. (2003) Actinomycetes in lake Ontario: habitats and geosmin and MIB production, Journal American Water Works Association, Vol. 95 (2): 113-118.

Zhu H.H., Guo J., Yao Q., Yang S.Z., Deng M.R., Phuong L.T.B., Hanh V.T. and Ryan M.J. (2007) *Streptomyces vietnamensis* sp. nov., a streptomycete with violet–blue diffusible pigment isolated from soil in Vietnam, International Journal of Systematic and Evolutionary Microbiology,Vol.57 (8): 1770–1774.

Williams S.T., Sharpe M.E. and Holt J.G. (1984 - 1989) Bergey's Manual of Systemic Bacteriology, Ed: William R. Hensyl, Williams and Wilkins, Baltimore, ISBN 0-683-09061-5, Vol. 4.

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