Eurasian Journal of Analytical Chemistry
ISSN: 1306-3057 OPEN ACCESS 2017 12 (7): 1045-1054

Cellulase Production from Polychaete Associated Bacteria, *Lysinibacillus Sphaericus*

R. Balasubramanian, R. Babu

Received 5 June 2017 ▪ Revised 7 August 2017 ▪ Accepted 17 September 2017

Abstract: **Background/Objectives:** Cellulases have a wide scope of utilizations. The principle potential utilizations are in animal feed, textile industry, food applications, fuel and chemical industries. Different regions of utilization incorporate the paper and pulp industry; squander the board, medicinal/pharmaceutical industry, protoplast generation, genetic engineering and pollution treatment.

Methods/statistical analysis: The components like temperature, pH, saltiness, substrate, and substrate fixation numerous significant for the production of cellulolytic enzymes were advanced by receiving seek strategy (i.e) fluctuating parameter each one in turn.

Findings: The ideal pH for the development and the enzymeactivity was observed to be at pH 7,at which growth was found to be 3.15 OD and enzyme activity was about 40U/ml/min. The optimum temperature for growth was found at 35ºC. Growth at 600 nm found to be 3.42 OD which was observed at 36 hrs and the enzyme activity was endowed to be 35U/ml/min. The maximum growth and the activity wasfound in corn cobs and the enzyme activity were about 40U/ml/min. The 50% of ammonium sulfate gave the most extreme precipitation of protein (0.84g). The dialysis membrane is partially permeable and has amolecular weight cut off between 12,000-14000. Polyacrylamide gel analytic test was utilized to decide the molecular weight of the readied enzymes. The analysis was completed under non-denaturing conditions for cellulase assurance.

Application: Cellulases have an extensive scope of potential applications, however impressive future research exertion is important to misuse the business capability of cellulases to the furthest reaches. Hence the present study on bioprospecting of polychaete associated bacterium covering cellulase production.

Keywords: Polychaaete, Bacteria, Lysinibacillussphaericus, optimization, Substrate, Mass-culture, Cellulase enzyme, Enzyme activity, Enzyme purification, SDS – PAGE.

INTRODUCTION

Cellulose is the most unlimited sustainable natural product in the biosphere (Solomon *et al*., 1997 and Whitaker, 1998). It is made out of long chains of D-Glucose molecule particles connected in beta-1, 4 configurations. Cellulase has the potential for production of a variety of useful compounds from cheaper and complex substrates. It is an important extra cellular microbial enzyme which hydrolyzes cellulose. From the inexpensive sources of valuable compounds such as sugar, protein and other chemicals are produced by enzymatic bioconversion. Yearly output of cellulose production is assessed to be 4.0×107 tons (Singh and Hayashi, 1995). The extent of cellulose in plant tissues ranges from 20 to 45% of dry weight and over 90% in cotton fiber (Stephens and Hatchel, 1995). Squander paper is a significant wellspring of cellulose (Crueger and Crueger, 1990).

Celluase is delivered mainly by bacteria, fungi and protozoan. Various biomass transformation strategies have been proposed and utilized running from direct chemical substance techniques like acid

R. Balasubramanian, Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai, Tamil Nadu, India. E-mail: balaram_r2@rediffmail.com
R. Babu, Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai, Tamil Nadu, India.

hydrolysis and pyrolysis to biological techniques, for example, use of cellulase enzyme protein (Cooney et al, 1978).

Cellulaseproduction has been amassed for the most part in fungi yet there is an expanding enthusiasm for cellulose production by bacteria as of late (Crawford, 1986 and Li and Gao, 1996). Acid hydrolysis of cellulosic materials is less expensive than cellulase hydrolysis however previous strategic methods regularly need high temperature and pressure. In addition, it is profoundly destructive and prompts aggregation of offensive results (Fenningtonet al., 1982).

MATERIALS AND METHODS

Optimization for Growth and Cellulase Production

Polychaete associated bacterium, *Lysinibacillussphaericus* was isolated and recovered for cellulase enzyme studies. The factors like temperature, pH, saltiness, substrate, and substrate concentration many important for the production of cellulolytic enzymes were optimized by adopting search technique (i.e) fluctuating parameter each one in turn. The experimental tests were directed in 250ml Erlenmeyer flasks containing production medium. After sterilization via autoclaving, the flasks were cooled and immunized with cultures at 37ºc for 72 hrs under different exploratory conditions as depicted below.

MEDIUM COMPOSITION (g/100ml)

$MgSo_4$	- 0.01
$(NH4)_2\ So_4$	- 0.02
KH_2Po_4	- 0.7
K_2HPO_4	- 0.05
Cellulose	- 0.1
Agar	- 1.5
pH	- 7.1 ± 0.2 at 25^{0}C

Optimization of Physical Parameterd for Growth and Cellulase Production

The fermentation was completed at various temperatures like 25ºC, 30ºC, 35ºC, 40ºC and 45ºC to think about their impact on growth and enzyme production. All of the above examinations were completed and normal qualities were calculated. Optimizationof pH, basal medium was varied from 3,5,7,9 and11 with 1N HCl and 1N NaOH. Every one of the analyses was done and normal qualities were determined. The medium was prepared with different salt concentration in the range of 1%, 2%, 3%, 4% and 5%. Each of the examinations was done and normal qualities were determined.

Effect of Different Substrate on Cellulase Production

The impact of different substrate on cellulose production was studied with different substrate like corn cobs, rice bran, wheat bran, cellulose and carboxymethylcellulose. Every one of the analyses was done and the normal qualities were reported. The impact of different substrate concentration on cellulase production was studied for maximum enzyme activity ranging from 1% to5%. Every one of the investigations was completed and normal qualities were calculated. The optimum substrate concentration accomplished by this progression was fixed in consequent investigations.

Mass Culture of Cellulase Producing Bacteria, Lysinibacillus Sphaericus

In light of the outcomes acquired through the enhancement, the mass scale culture of the cellulose producing from Lysinibacillussphaericus was done dependent on the outcomes got. The copy 500ml of production media was inoculated with 5% of inoculums. The fermentation was completed in 1000 ml Erlenmeyer flasks on a rotational shaker (300 rpm). The biomass and the protein enzyme activity were tried at each 6 hrs interim. Toward the finish of the 36th hour the way of culture was harvested for the recuperation of cellulaseenzyme.

Enzyme Activity

Cellulaseactivity was estimated by DNS technique, through assurance of decreasing sugars freed (Krootdilaganandh, 2000). 0.5 ml of CMC solution, 0.5 ml of rough enzyme and 0.5 ml of 0.05 M citrate buffer pH 4.8 were incubated for 30 min at 50o C before including 2 ml of DNS solution. The treated examples were boiled for 15 min. before chill off in cold water for color adjustment. The optical density was perused at 540 nm against reagent clear by a spectrophotometer (Thermo Spectronic, USA).

Extraction and Partial Purification of Cellulase Enzyme

The various steps were followed for enzyme purification, was done at 4ºC except if generally expressed. In the underlying decontamination step, the supernatant containing the extracellular enzyme

enzyme was treated with different saturation levels of solid ammonium sulphate (40%,60% and 80% saturation level), with continuous overnight stirring (Wang et al.,2006). The precipitated enzyme was collected by centrifugation (10,000 rpm for 15 min) and diffused in 0.1M citrate phosphate buffer (pH 5.0). The enzyme solution was dialyzed in a dialysis membrane No.150 (HIMEDIA) against the same buffer for 48hrs with several intermitter buffer changes. The partially purified enzyme obtained was lyophilized in to a powder and the cellulase activity was assayed.

Determination of Molecular Weight by Electrophoresis (SDS-PAGE)

Electrophoresis of protein tests in the polyacrylamide gel is an imperative scientific and now and again, preparative apparatus for the protein researcher. Electrophoresis can be utilized to separate and analyze complex protein blends, assessed immaculateness of a protein over the span of its isolation, and give appraisals of physical attributes, for example, subunit composition, isoelectric point, size and charge. Each kind of electrophoretic separation can be led in an assortment of gel sizes extending from micro gels just marginally bigger than a postage stamp to monster gels a lot bigger than this page. SDS-based gel separations are vigorous as most yet not all proteins are promptly solubilized in SDS solutions. Most proteins tie a uniform measure of SDS per microgram of protein which gives a uniform charge density for each unit mass to give a partition dependent on the mass of the polypeptide chain.

After electrophoretic separation, proteins are typically identified utilizing general protein stainning techniques, for example, coomassie blue staining or the more sensitive silver staining. These staining strategies fix the proteins in the gel matrix by either chemical cross-linking or denaturation to forestall resulting dissemination of the protein bands. Electrophoresis is utilized to isolate complex blends of proteins to examine subunit structure to check homogenicity of protein tests and to filter proteins for use in further applications in polyacrylamide gel electrophoresis (PAGE), proteins migrate because of an electric field through pores in the gel matrix. The migrate rate of protein is dictated by the gel pore size and the protein charge, size and shape in Sodium-dodecylsulphate-polyacrylamide gel electrophoresis.

1. SDS-PAGE of proteins

Acrylamide and N, N-methylene bisacrylamide. A stock solution containing 29 % (w/v) acryl amide and 1%(w/v) N,N-methylene bis acryl amide was prepared in deionized water.

2. SDS (Sodium-dodecylsulphate)

A 10% (w/v) stock solution was prepared in deionized water and kept in room temperature

3. SDS Discontinuous buffer stock 6g of Tris was dissolved in 40ml of deionized water and then titrate to pH 6.8 with 1M Hcl and made upto 100ml with deionized water.

4. TEMED (N, N, N, N-Tetramethylethylenediamine) Used as supplied.

5. APS (Ammonium per sulphate) A 10% (w/v) stock solution was prepared freshly in deionized water and kept at 4°C until use.

6. Reservoir buffer or Tris glycine electrophoresis buffer. This buffer includes: 25mN Tris base, 250mM glycine pH8.3 (electrophoresis grade), 0.1% SDS water. At that point, 50ml of 10% (w/v) stock solution of SDS was filled and the volume was fine-tuned to 1000ml with water.

7. SDS Reducing sample buffer

SDS	1g
0.5M Tis Hcl (pH6.8)	2.5ml
Sucrose	3g
B-Mercaptoethanol	1ml

This was made upto 10ml with deionized and stored at 4°C.

8. Staining Solution

Coomassie Brilliant Blue Stain	100mg
Methanol	40m
Glacial acetic acid	10ml
Water	50ml

9. Destaining Solution

Methanol	40ml
Glacial acetic acid	10ml
Water	50ml

The samples were solubilized in reducing sample buffer and equivalent measure of protein was stacked into 12% SDS-Polyacrylamide gel and electrophoresis was completed at consistent current (30 mA).

RESULTS

*Lysinibacillussphaericus*strain showed a 12mmclearence in cellulose agar medium.

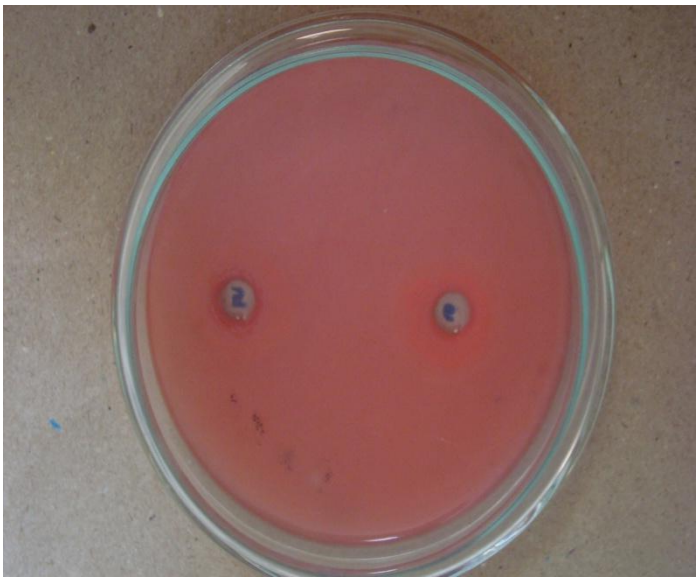

Fig. 1: Zone of clearance in the Cellulose Agar medium

Optimization of Growth and cellulase Production pH

Among the various pH studied the optimum pH for the growth and the enzyme activity was found to be at pH 7, at which growth was found to be 3.15 OD and the enzyme activity was about 40U/ml/min.

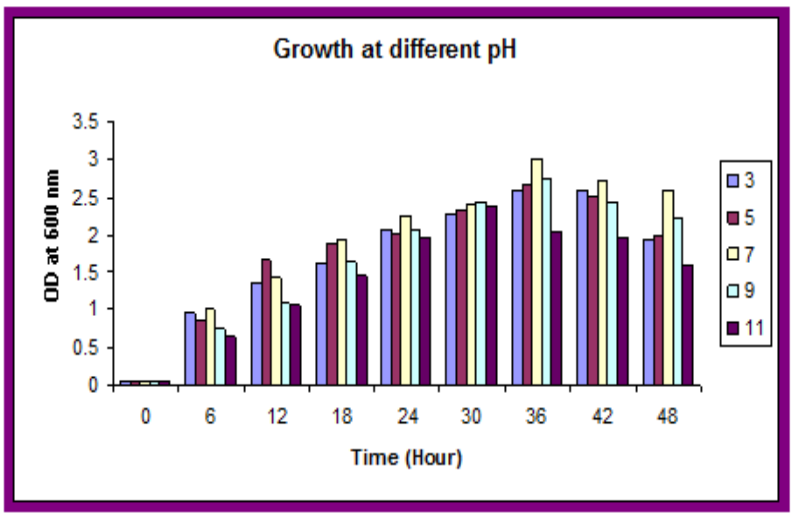

Fig.2: Bacterial growth at different pH

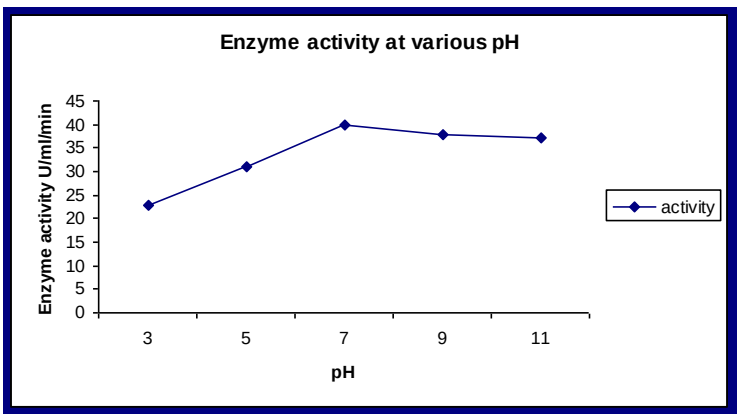

Fig.3: Enzyme activity at various pH

Optimium Temperature for Growth and Cellulase Production

Among the different temperature studied, the optimum temperature for growth was found at 35ºC. Growth at 600 nm found to be 3.42 OD which was observed at 36 hrs. The enzyme activity was observed to be 35U/ml/min.

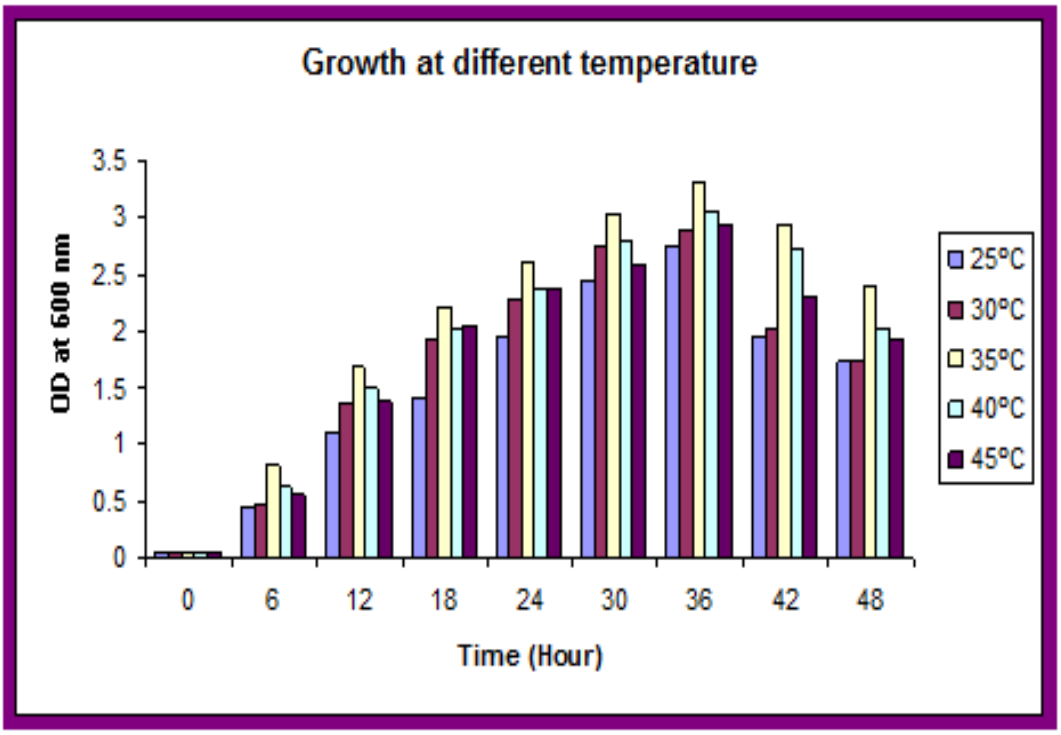

Fig.4: Bacterial growth at different temperature

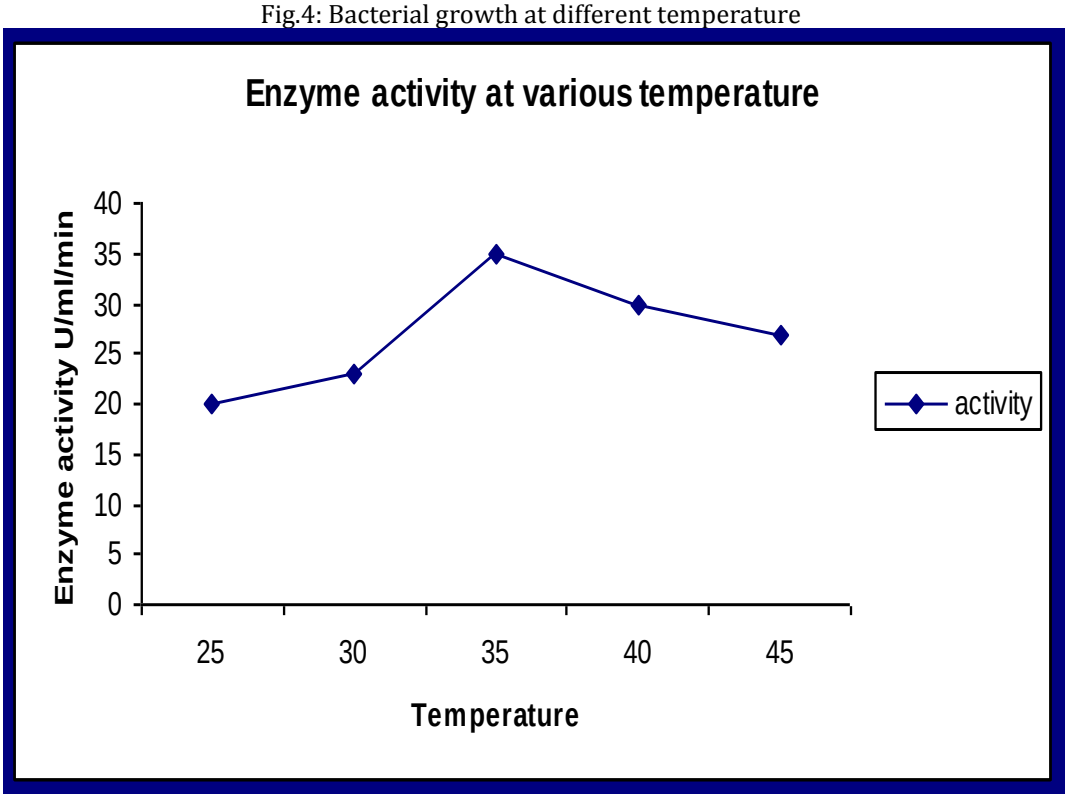

Fig.5: Enzyme activity at Various Temperature

Optimum Salinity for Cellulase Production

Amongst the different temperature studied, the optimum temperature for growth was found at 35ºC. Growth at 600 nm found to be 3.42 OD which was seen at 36 hrs. The enzyme activity was observed to be 35U/ml/min.

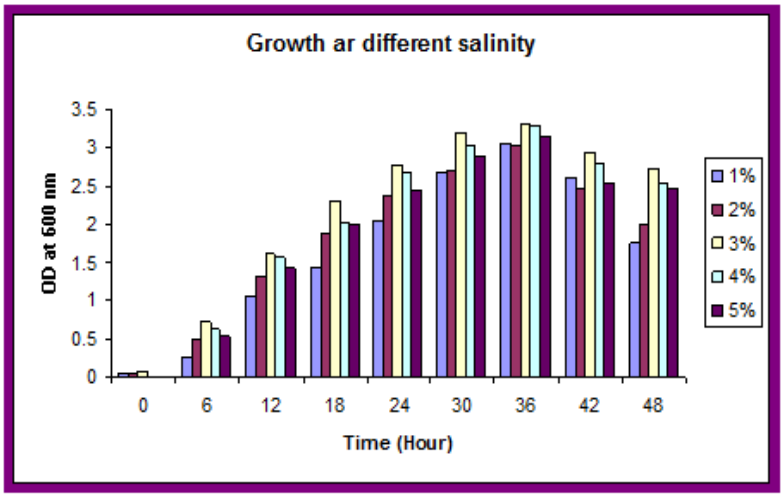

Fig.6: Bacterial growth at different salinity

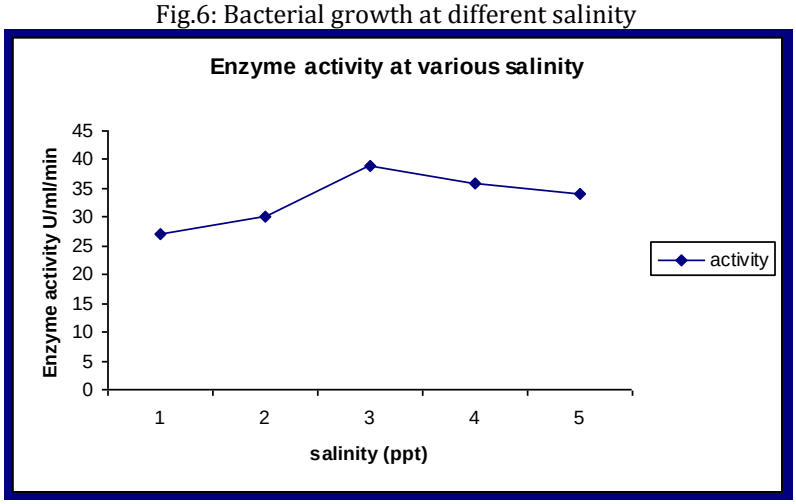

Fig. 7: Enzyme activity at various Salinity

Optimum Substrate for Cellulase Production

Among the different substrates used the maximum growth and the activity wasfound in corn cobs. The enzyme activity was about 40U/ml/min. and the maximum growth was found to be 3.63 OD.

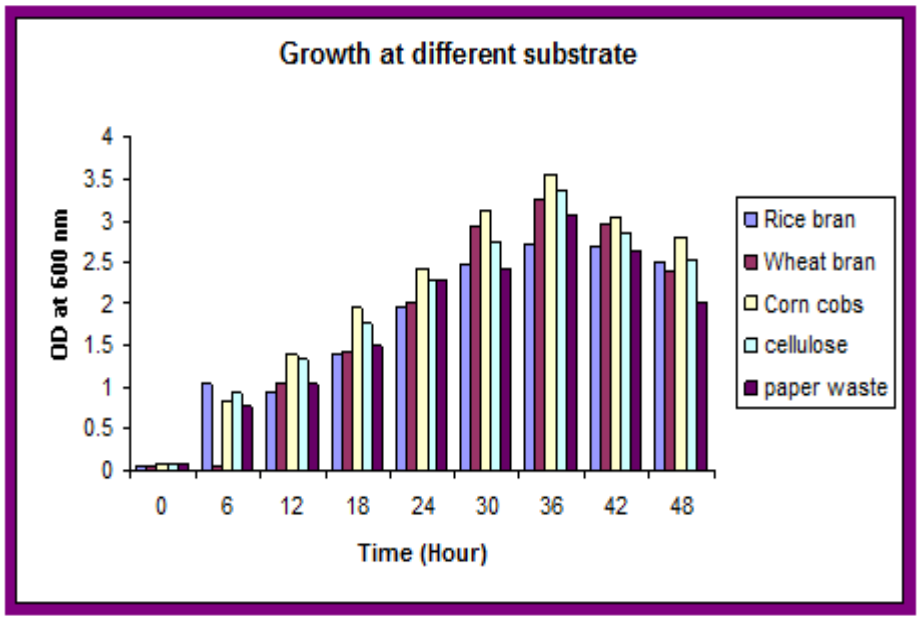

Fig.8: Bacterial growth at different substrates

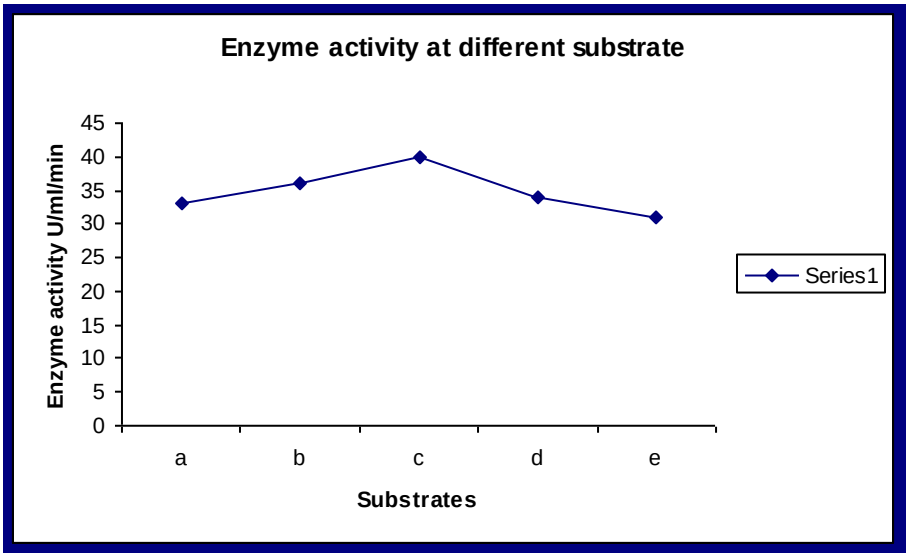

Fig.9: Enzyme activity at different substrates

Optimum Substrate Concentration for Cellulase Production

Among the different substrate concentration used the maximum growth (3.75 OD) and the enzyme activity was found at 3%. The Enzyme activity was observed to be 43U/ml/min.

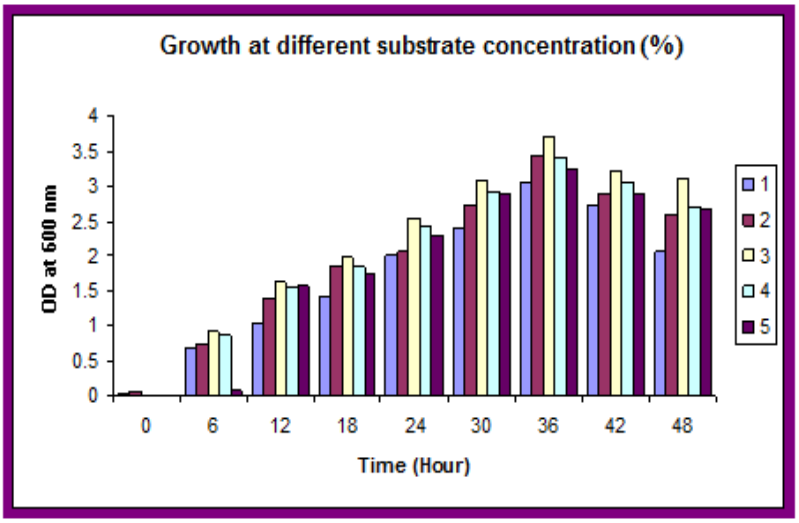

Fig.10; Bacterial growth at different corn cobs concentration (%) as substrate

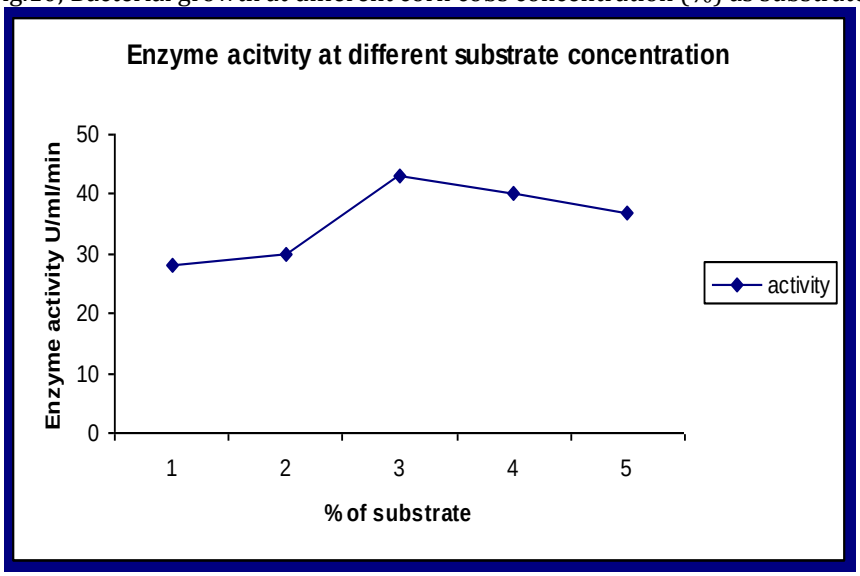

Fig.11: Enzyme activity at Different substrate concentration

Ammonium Sulphate Precipitation for the Partial Purification of Extracellular Cellulase Enzyme

The most potential strain *Lysinibacillussphaericus*was selected for mass culture using the optimized condition. The 500ml of cell free supernatant was obtained. The extracellular cellulose enzyme was precipitated with changing measure of ammonium sulphate (30%, 40%, half, 60% and 70%).The half of ammonium sulphate gave the greatest precipitation of protein (0.84g) pursued 40% (0.56g) and 30% (0.17g). The purification began with the dialysis in a recovered consistent cellulose tube against phosphate buffor for 24-48 hours. The dialysis membrane is in part porous and has a molecular weight cut off between 12,000-14000.

Molecular Weight Determination by Sds-Page Analysis

Polyacrylamide gel analysis test was utilized to decide the molecular weight of the readied enzymes. The analytic test was completed under non-denaturing conditions for cellulase assurance.

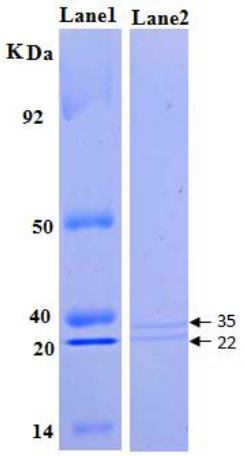

Fig. 12: SDS-PAGE of purified cellulase

The outcomes displayed that the purified cellulase enzyme has a molecular weight range from 22 to 35 kDa.

DISCUSSION

In the present study *Lysinibacillussphaericus*strain showed 12mm of zone of clearance in congo red test. Screening of bacterial microbes was led by utilizing the Congo red test as a primer report for choosing cellulase producers In another study about 9 bacterial strains (EB1-EB9) showed positive result in congo red test, of which EB3 demonstrated the most elevated proportion of clear zone diameter to colony diameter across showing more cellulose debasement in CMC agar plates (Ariffin,2006). Salinivibriosp. NTU-05, detached from Szutsausaltern showed apparent clear zones around the colonies on CMC agar plates following staining with 1% Congo red solution, demonstrating that it secretes conspicuous quantity of cellulose (Wang, 2009)

In the present research medium optimization for cellulose production was completed utilizing diverse parameters like pH, temperature, saltiness, substrate and substrate concentration. The ideal pH for cellulose production was observed to be pH 7, and the equivalent was seen in purification and portrayal of a novel halostablecellulase from Salinivibriosp. strain NTU-05 which held the most extreme enzyme activity at pH 7(Wang,2009).In the purification and portrayal of CMC isolated from a marine bacterium, Bacillus subtilis subsp.subtilisA-53 the ideal pH for the CM Case activity was observed to be at 6.5(Kim,2009).

Among the different substrates used corncobs showed increased enzyme activity and the optimum substrate concentration was found to be 3%. *Bacillus subtilis* showed the maximum enzyme activity when grown on the increased pineapple peel substrate concentration (10%) (Sunitha,2006).Molecular weight determination of cellulase was carried out using SDS-PAGE analysis. In the present research study the outcomes displayed that the purified cellulase protein enzyme had a molecular weight scope of 22-35 kDaPurifiedCM Case from *Bacillus spp*has a molecular weight range of 30-65 kDa (Ariffin, 2006). The molecular mass of the bacterial strains, CH43 and HR68 estimated from SDS-PAGE was 40kDa

(Mawadza,2000). A halostablecellulase with an atomic mass of 29 kDa was purified utilizing SDS-PAGE (Wang, 2009).

The Lysinibacillus sphaericus used in this research study delivered promising amount of cellulase and can consequently expand the cellulase maker families into the genus Bacillus. In another study mutant of Bacillus (BpCRI 6) exhibited a few preferences, for example, high activity in nearness of substrates like CMC and cellobiose (Kotchoni, 2003). Two bacterial species, Acinetobacteranitratus and Branhamellasp., were confined from the haemolymph of the goliath African snail, Archachatinamarginata. The endocellulase activity of the way of culture broth was resolved amid bacterial development by estimating the arrival of decreasing sugar from carboxymethyl cellulose (CMC)(Ekperigin, 2007). Vetriselvi (2007) isolated five species of bacteria namely *Pseudomonas, Proteus, Micrococcus, Serratia and Bacillus* and five species of fungi viz., *Aspergillusniger, Trichodermaharzianum, Myrotheciumroridum, Curvularialunata* and *Fusariumoxysporum*isolated from agricultural crop and their cellulolytic activity was studied.

A total of 15 isolates were obtained during the course of investigation from biodegrated leaf litter compost of which *Bacillus, Cellulomonas* and *Pseudomonas* revealed as fast growing bacterial isolates.Three species of *Bacillus* namely *B.subtilis, B.licheniformis and B.polymyxa* degrading cellulose were reported by Majumdar (2001). Making of cellulase from As pergillusniger and Bacillus subtilis using pine apple strip as substrate by strong state fermentation. was carried out in which the maximum enzyme yield was obtained in*B.subtilis*when compared to*A.niger*(Sunitha,2006).A study on examination of oxygen consuming cellulolytic microorganisms in some north timberland and cultivating soils was conducted and found that the level of cellulolytic microscopic bacterial organisms in woods soil was more when contrasted with cultivating soil tests (Hatami, 2008). Nitrogen fixing Paenibacillus strains were isolated from soil in N-free media. Three of these cellulase positive detaches with CMCase were distinguished as Paenibacillus strain E, H and SH (Emtiazi, 2007).

CONCLUSION

Cellulases have a wide scope of prospective applications, however significant future research exertion is important to abuse the commercial capability of cellulases to the furthest reaches. In this present study the strain was used produced maximum activity of 45U/ml/min. which seemed to be in appreciable range. The physiochemical parameters optimum for the growth and enzyme production showed that the strain used was ideal for industrial scale production.

REFERENCES

[1] Ariffin, H., N. Abdullah, M.S.U. Kalsom, Y. Shirai and M.A. Hassan, 2006.Production and characterization of cellulase by *Bacillus pumilus* EB3.*Int. J. engg. tech*., 3(1): 47-53.

[2] Cooney CL, Wang DIC, Wang SD, Gordon J, Jiminez M (1978). Simultaneous cellulose hydrolysis and ethanol production by a cellulolytic anaerobic bacterium.Biotechnol.Bioeng.Symp. No. 8: 103-114.

[3] Crawford DL (1986). The role of actinomycetes in the decomposition of lignocelluloses. FEMS Symposium pp. 34-728.

[4] Crueger W, Crueger A (1990). Biotechnology.A textbook of Industrial Microbiology 2nd ed. Science Technology Publishers, USA.p. 357.

[5] M.M. Ekperigin. 2007. Preliminary studies of cellulase production by Acinetobacteranitratus and Branhamella sp. African Journal of Biotechnology Vol. 6 (1), pp. 028-033.

[6] Emtiazi, G., M. Pooyan and M. Shamalnasab, 2007.Cellulase activities in nitrogen fixing *Paenibacillus* isolated from soil in N- free media. *W. J. Agri. Sci.,* 3(5): 602-608.

[7] Fennington G, Lupo D, Stutzenberger F (1982). Enhanced Cellulase Production in Mutants of Thermomonosporacurvata. Biotechnol.Bioeng. 24: 2487-2497.

[8] Hatami, S., H.A. Alikhani, H. Besharati, N. Salehrastin, M. Afrousheh and Z. Y. Jahromi, 2008.Investigation on aerobic cellulolytic bacteria in some of north forest and farming soils. *J. Agri. Environ. Sci.,* 3(5): 713-716.

[9] Kim, B.K, B.H. Lee, Y.J. Lee, I.H. Jin, C.H. Chung, J.W. Lee, 2009. Purification and characterization of carboxymethylcellulase isolated from a marine bacterium, *Bacillus subtilissubsp. subtilis. Asian. J. Microbiol. Biotech.,*53: 220-224.

[10] Kim, J. H., H.U. Dahms, J.S. Rhee, J.M. Lee, J. Lee, K.N. Han and J.S. Lee, 2010. Expression profiles of seven glutathione S-transferase (GST) genes in cadmium-exposed river pufferfish (*Takifuguobscurus*). *Comparative Biochem. Physiol. Part C*, 151: 99-106.

[11] Kotchoni, O.S., Shonukan O.O. and Gachomo, W.E. (2003) Bacillus pumillus, BpCRI 6, a promising *O.S. Kotchoni, O.O. Shonukan, W.E. Gachomo.* 2003. *Bacillus pumilus BpCRI 6, a promising candidate for cellulase production under conditions of catabolite repression*. African Journal of Biotechnology: 2(6): 140-146.

[12] Kotchoni, O.S., Shonukan O.O. and Gachomo, W.E. (2003) Bacillus pumillus, BpCRI 6, a promising candidate for cellulase production under conditions of catabolite repression. African Journal of Biotechnology, 2(6): 140-146

[13] Kotchoni, O.S., Shonukan O.O. and Gachomo, W.E. (2003) Bacillus pumillus, BpCRI 6, a promising candidate for cellulase production under conditions of catabolite repression. African Journal of Biotechnology, 2(6): 140-146

[14] Krootdilaganandh, J., 2000. Isolation and selection of thermotolerant bacteria capable of producing cellulase. Chiang Mai: Chiang Mai University Press, p20-21.

[15] Li, M., H. Yang and J. Gu, 2009. Phylogenetic diversity and axial distribution of microbes in the intestinal tract of the polychaete. *Neanthesglandicincta*, 12: 892–902.

[16] Majumdar P, Chanda S, Majumdar P, Chanda S. Chemical profile of some lignocellulosic crop residues. Indian J AgrBioch. 2001;14:29–33.

[17] Singh, M.P.N., J. Ahmed and M.P. Sinha, 2001.Effect of cellulose (a polysaccharide) on secretion of pectolytic and cellulolytic enzymes by blight pathogens.*Asian J. Microbiol. Biotech.,* 3(4): 311-314.

[18] Singh A Hayashi K (1995). Microbial Cellulases. Protein Architecture Molecular Properties and Biosynthesis. Adv. Appl. Microbiol. 40: 1-35.

[19] Solomon BO, Amigun B, Betiku E, Ojumu TV, Layokun SK (1997). Optimization of cellulase production by Aspergillusflavus Linn isolate NSPRI101 grown on bagasse. J. Nig. Soc. Chem. Eng. 16: 61-68.

[20] Stephens GR, Heichel GH (1975). Agricultural and forest products as sources of cellulose. Biotechnol.Bioengr.Symp. 5: 27-42.

[21] Sunitha, M., E. Rani and K. Devaki, 2006. Production of cellulase from *Aspergillusniger* and *Bacillus subtilis* using pine apple peel as a substrate by solid state fermentation. *Asian J. Microbiol.Biotech.,* 8(3): 577-580.

[22] Vetriselvi, J., C. Muthukumar, G. Jayanthi and R. Saravanamuthu, 2007. Studies on the potentiality of cellulose degradation by the micro organisms associated with crop residues in the soil. *Asian J. Microbiol.Biotech.,* 9(1): 91-94. *Invertebr. Biol*., 116:102–114.

[23] Wang, C. Y., Y. R. Hsieha, C.C. Nga, H. Chanb, H. Linc, W.S. Tzenga and Y.T. Shyua, 2009. Purification and characterization of a novel halostablecellulase from *Salinivibrio* sp. strain NTU-05. *Asian J. Microbiol.Biotech.,*7*:* 311-315.

[24] Wang.l, Zhng. Y, Gao.P, Shi. D, Liu.H and Gao.H, 2006.Changes in the structural properties and rate of hydrolysis of cotton fibers during extended enzymatic hydrolysis. Biotechnol. Bioeng., 20; 93(3): 443-56.

[25] Whitaker JR (1990). Cellulase Production and Applications. Food Biotechnol. 4: 669-697.