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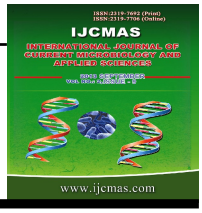

Original Research Article

Cellulase from an estuarine *Klebsiella ozeanae*

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A B S T R A C T

To produce cellulase using solid state fermentation from *Klebsiella Ozeanae* cellulase producing bacterial population was isolated from water and sediment samples and plated on to cellulase agar medium. Among the 8 strains, *Klebsiella ozeanae* exhibited highest zone of lysis. Hence, it was selected for further production, optimization incubation period, temperature, pH, salt, nitrogen sources carbon source. The results showed that the optimum conditions for maximum celllulase production under SSF (Solid State Fermentation) by *Klebsiella Ozeanae* was found to be on the second day of incubation, optimization incubation period 48 h, temperature 35°C, pH 7, salt 3%, nitrogen sources peptone carbon source glucose.) and characterization of cellulase enzyme Fractional precipitation of proteins with varying saturation levels of ammonium sulfate was used as the first step in the separation and purification of cellulase enzyme from the mass culture of *Klebsiella ozeanae* grown in cellulose production broth and the cellulase enzyme molecular weight determination(32 kDa). Cellulase production by Estuarine *Klebsiella ozeanae* was significantly enhanced by optimization of medium composition and culture conditions. Given that cellulase is a natural product of bacteria in the marine environment, and that its presence enhances the bacterial use in ethanol production from cellulose

K e y w o r d s

Isolation; Sceening; Otimization; Purification; SDS PAGE; cellulase enzyme; *Klebsiella ozeanae*.

Introduction

Enzymes are biocatalysts which accelerate biological reactions. Enzymes are biocatalysts as they are molecules secreted by living cells as well as they are accelerating the biological reactions. Compared to the other sources, microbial sources have gained much popularity. At present, more than 2000 enzymes have been isolated and characterized, out of which 1,000 enzymes were recommended for various applications. About 50 microbial enzymes have industrial application. Cellulose is the most abundant renewable natural product in the biosphere (Qin *et al.*, 2010). It is composed of long chains of D- Glucose molecules linked in beta- 1, 4 configurations. Annual production of cellulose is estimated to be 4.0×10^7 tons (Singh and Hayashi, 1995). Many crucial details of cellulose

hydrolysis are still to be uncovered. Yet, a mechanistic model for the action of enzyme complexes on the surface of insoluble substrates becomes apparent and the application of enzymatic hydrolysis of cellulosic biomass can now be addressed (Schwarz *et al.*,, 2001.) Celluloses are regarded as the most important renewable resource for bioconversion. Many Cellulosic substances were hydrolyzed to simple sugars for making Single Cell Protein, sweeteners etc. (Bai *et al* ., 2012) . Nowadays enormous amount of agricultural and industrial cellulosic wastes have been accumulating in environment. Celluloses are regarded as the most important renewable resource for bioconversion. The proportion of cellulose in plant tissues ranges from 20 to 45% of dry weight and over 90% in cotton fiber (Waste paper is an important source of cellulose). Due to the crystalline structure of filter paper, degradation of the filter paper would imply multiple cellulase activities including exoglucanase activities because these enzymes work in crystalline regions (Dashtban *et al.,* ,2010) The enzyme cellulase is useful in converting the plant biomass in to fuels and basic chemicals and many other useful products (Lopez *et al.,* , 2000.) Celluase is produced chiefly by bacteria, fungi and protozoan (Pothiraj *et al* ., 2006). A number of biomass conversion methods have been proposed and employed ranging from direct chemical methods like acid hydrolysis and pyrolysis to biological methods such as application of cellulase enzyme. Cellulose decomposing bacteria and fungi are widely distributed in the marine environment and they play an important role in mineralizing organic matter and also influencing the productivity of the sea (Lynd *et al.,* , 2002). Cellulose production and found to produce a variety of unique cellulases including some of which were found to be thermoalkotolerant(George *et al.,* ,2010) Compared to terrestrial Streptomycetes, many of the marine isolates are found to be active cellulose decomposers. The catalytic domains of EngB and EngD formed inclusion bodies when expressed in *E. coli*. On the other hand, both catalytic domains containing the C-terminal cellulose-binding domain (CBD) of EngD were expressed in soluble form (Murashima *et al.,* ,2003). (Nishiyama *et al* ., 2003) Crystalline or native cellulose is hydrolysed to its component glucose units by the combined activities of endo beta 1, 4-glucanase, cellobiohydrolase (CBH) and beta-1,4-glucosidase, and endo beta-1 ,4 glucanase which randomly hydrolyses internal beta-1, 4-glycosidic bonds of cellulose polymers of 4 or more glucose units. The use of cellulosic biomass as a renewable source of energy via breakdown to sugars that can then be converted to liquid fuel is of great interest (Pason *et al.*,,2010). Numerous investigations have reported the degradation of cellulolytic materials, but few studies have examined which microorganisms had met the industrial requirement. So there, new industrial relevant cellulolytic and hemicellulolytic enzymes are being considered (de Castro *et al.,* , 2010). Research and development to reduce the cost of bioethanol has been carried out in various aspects. Recently, the cost of ethanol production from cellulosic material is US$1.8 per gallon. However, development of enzymatic processing can decrease the ethanol cost as low as US$0.2 per gallon (Genansounou *et al.,* , 2010). Cellulose is the structural component of the primary cell wall of green plants, many forms of algae and the oomycetes. Some species of bacteria secrete it to form biofilms.(Verma *et al* ., 2012).

Materials and Methods

Isolation of cellulase Producing Bacteria

Study area

Uppanar estuary

The Uppanar estuary is situated at Cuddalore in Tamil Nadu,India which is about 25 km away from the Parangipettai coast. It is formed by the confluence of Gadilam and Paravanar rivers. Uppanar estuary is also an open type estuary and the width of the mouth is around 30 m . SIPCOT industrial park (State Industries Promotion Council of Tamil Nadu) is located on the northern bank of Uppanar estuary covering an area of about 520 acres with 44 industries, which include chemicals, petrochemicals, pharmaceutics, pesticides, fertilizers and metal processing industries. The former originates from the foothills of northeastern part of the Shervarayan hills and later from Vridhachalam Taluk of Cuddalore district.

Collection of samples

Water, sediment samples were collected from the Uppanar estuary. 1g/1ml of sample was suspended in 99 ml sterile 50% aged sea water, agitated for 45min in a shaker at 50^0C and 0.1 ml was spreaded on cellulase agar plates $MgSO_4$ - 0.01g, $(NH_4)_2SO_4$ - 0.02g KH_2PO_4,- 0.7g, K_2HPO_4- 0.05g, Cellulose- 0.1g. 50% aged seawater - 100ml and incubated at 30^0C for 48hrs (Ekperigin, 2007).

Screening for cellulase producing bacteria (well diffusion assay)

The isolated bacterial strains were inoculated into cellulose agar (1% carboxy methyl cellulose and 2% agar) with cellulose as the sole source of carbon. After an appropriate incubation was done at 28 ± 2^0C for 48 hours, the agar medium was flooded with a aqueous solution of Congo red (1mg/ml for media containing CMC) for 15 minutes. The Congo red solution was poured off, and plates were further treated by flooding with 1M NaCl for 15min. Based on the diameter of zone of clearance, the organism was selected for identification and further study.

Optimization of culture conditions for enzyme production

The culture conditions (incubation period, pH, temperature, salt concentration and different sources of C and N) were optimized for maximum enzyme production using cellulose producing medium. Cellulase production was studied at different pH (3-9 with 1 interval), temperature (25-50^0C with 5^0C interval), incubation period (24hrs -120hrs), salt concentration (2% to 7%), different carbon sources 1g (glucose, maltose, sucrose, raffinose, dextrose, fructose, starch and mannitol) and different nitrogen sources1 g (yeast extract, beef extract, peptone, Ammonium nitrate, Ammonium sulphate, Ammonium chloride). Enzyme production was followed up to 96 hrs. The medium was incubated at 30^0C for 72h in a shaker.

Result and Discussion

In the present study to isolate cellulase producing bacterial population water and sediment samples were plated on cellulase agar plates (pH 7) and incubated at room temperature at 28^oC. Bacterial density was found to be 2.4 x 10^8 CFU/g in sediment samples and 1.7 x 10^6 CFU/ml in water samples. Totally 8 strains were selected for the cellulolytic activity and they were identified upto species level as *Bacillus*

megaterium, B. subtilis, Citrobacter freundii, Corynebacterium xerosis, Corynebacterium sp., *Eubacteria aerogenes, Klebsiella ozeanae* and *Pseudomonas aeruginosa.*

All the 8 bacterial strains isolated belonging to ten genera were qualitatively assayed by well diffusion assay method in water agar medium supplemented with 1% cellulose. Among the 8 strains, *klebsiella ozeanae* exhibited largest zone of lysis (1.4 cm). Hence, it was selected for further production, optimization and characterization of cellulase enzyme. Incubating the *Klebsiella ozeanae* cultures in cellulase production broth at 200 rpm resulted in the production of 2.86 U/ml/min which seemed to be higher in 48 h incubation (Figure.1). The initial medium pH ranging from 3-9 was studied for their effect on cellulase production. The pH 7 supported the maximum cellulase activity as 2.92 U/ml/min and the minimum enzyme activity (1.86 U/ml/min) were observed in culture grown at pH 3 (Figure.2). Different temperatures varying from 20°C to 50°C were observed in the present study for the detection of optimum temperature required for the production of the cellulase enzyme. 35°C seemed to be the most favorable as the maximum production of about 3.36 U/ml/min was obtained and minimum cellulase activity was observed at 20°C (1.02 U/ml/min) (Figure.3). When eight different carbon sources were tried, the highest enzyme activity was obtained in cellulose amended medium (3.92 U/ml/min) and the minimum enzyme activity was found in glucose amended medium (1.82 U/ml/min) (Figure.4).

As cellulose gave maximum cellulase productivity, it was included as the sole carbon source in further optimization experiments. Different organic and inorganic nitrogen sources were studied in which peptone gave the maximum enzyme activity of about 4.01 U/ml/min at the 48h of incubation. Likewise, the minimum enzyme activity (2.73 U/ml/min) was observed with Di ammonium sulphate (Figure 5). As the bacterial strain was isolated from the estuarine environment, the effect of varying percentage of NaCl concentration on cellulase production was studied. At 3 % of salt concentration, maximum cellulase activity (4.19 U/ml/min) was observed. Likewise minimum enzyme activity was obtained at 1 % of salt concentration (2.32 U/ml/min) (Figure.6).

Fractional precipitation of proteins with varying saturation levels of ammonium sulfate was used as the first step in the separation and purification of cellulase enzyme from the mass culture of *Klebsiella ozeanae* grown in cellulose production broth with all the optimized nutritional and environmental conditions during which the enzyme activity was found to be 5.24 U/mg/min (Coral *et al.,* , 2002) . The centrifuged culture supernatant was used as the crude enzyme source for purification. During fractional precipitation, 60 % saturation of ammonium sulfate yielded maximum enzyme as studied in the qualitative plate assay for cellulase activity. Since the suspension from 60 % ammonium sulfate saturated culture filtrate exhibited maximum lytic zone of about 11 mm, it was used for the precipitation of proteins from the culture filtrate (Figure .7).

SDS PAGE

The samples were solubilized in reducing sample buffer and equal amount of protein was loaded into 12% SDS-Polyacrylamide gel and electrophoresis was carried out at

Figure.1 Optimization of incubation period

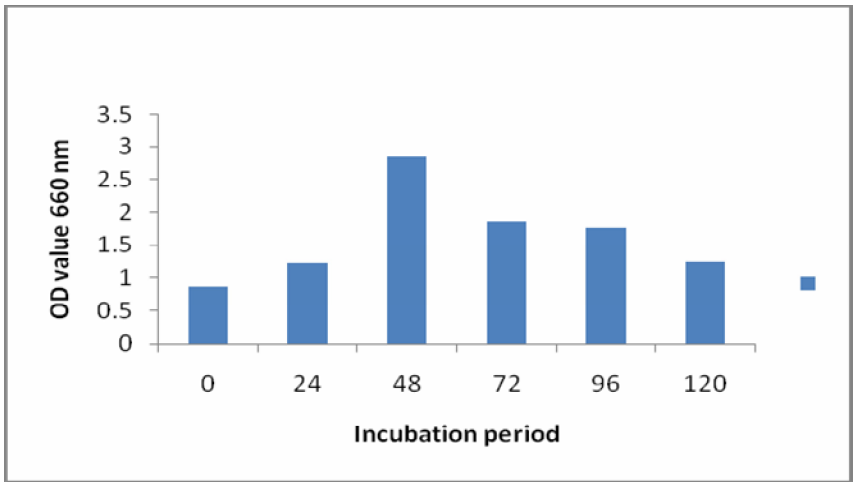


Figure.2 Optimization of pH

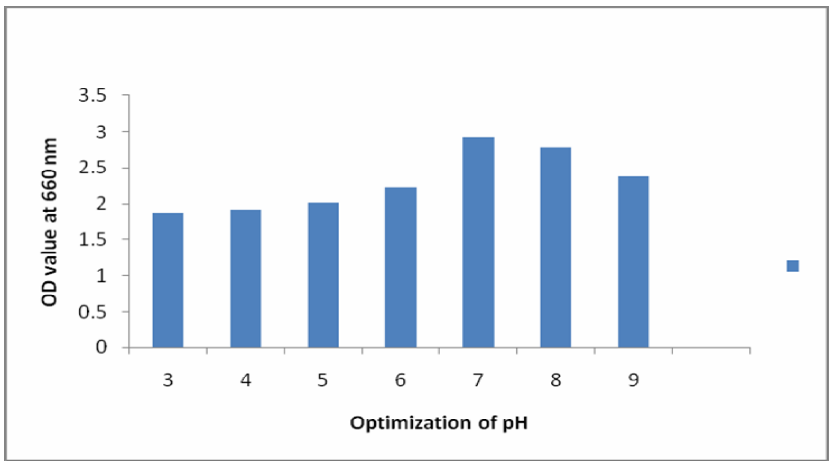


Figure.3 Optimization of Temperature

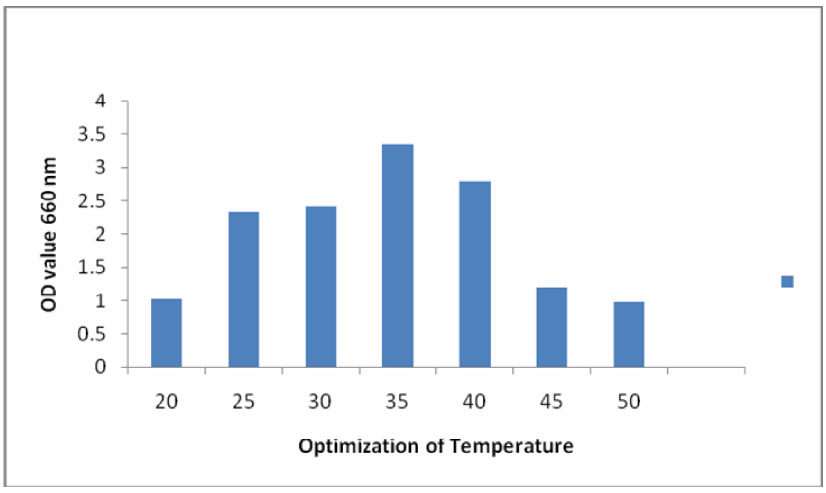


Figure.4 Optimization for carbon source

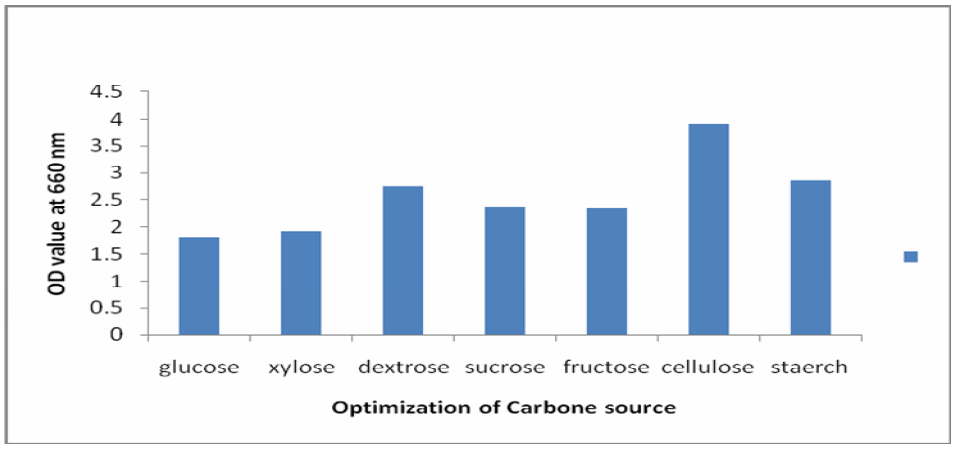


Figure.5 Optimization of nitrogen source

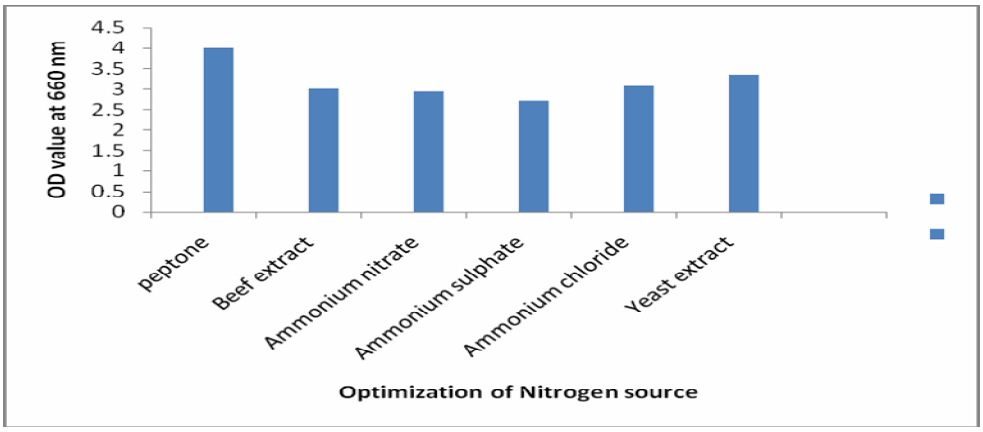


Figure.6 Optimization of salt concentration

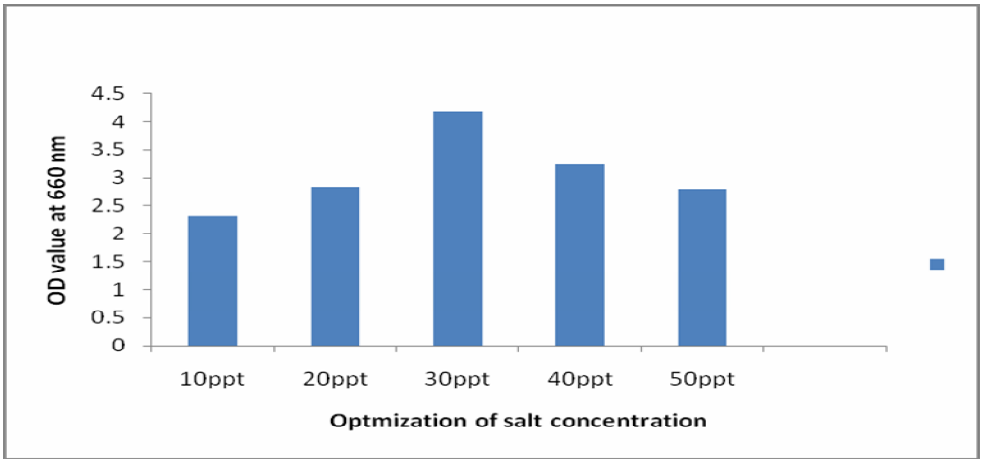


Figure.7 Ammonium sulphate precipitation

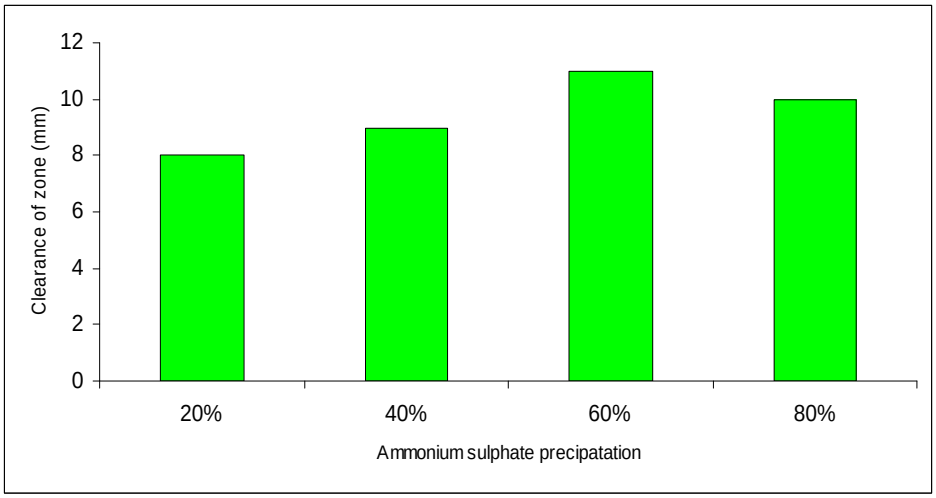
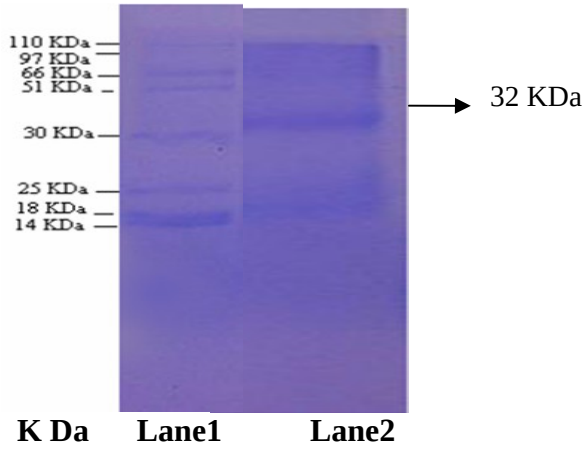


Figure.8 SDS-PAGE of Purified Cellulase



Lane 1: molecular weight markers, **Lane 2:** purified cellulose

constant current .In the present study the results exhibited that the partially purified cellulase enzyme had a molecular weight range of 32 kDa. The similar study reported from *Pasteurella multocida* has a molecular weight range of 32 kDa. A halostable cellulase with a molecular mass of 29kDa was purified using SDS-PAGE (Wang, 2009) (Figure 8).

The partially purified enzyme obtained was lyophilized in to a powder and the cellulase activity was assayed. The samples were solubilized in reducing sample buffer and equal amount of protein was loaded into 12% SDS-Polyacrylamide gel and electrophoresis was carried out at constant current (30mA). Using these techniques (Ibarra *et al.,* 2004) analyzed a more conventional protein with the two amide bands dominating the spectrum and only traces of polysaccharides and lignin as well as a cellulase produced by *Trichoderma* (Lee *et al.,* 2003).

The optimum incubation period for the present study was 48 h which is comparatively lesser (Ali *et al.,* 1991) and got the maximum cellulase production at 96 h. The present states that the bacterial strain *K. ozeanae* produces high cellulase production in the lower incubation period. In the present study it was observed that pH 7 was optimum for the maximum yield of cellulase production which is supported by the work (Akiba *et al.,* 1995). However (Xiaoting *et al.,* 2008) reported that maximum pH 7 was optimal for cellulase production. In the present study, 35^{o}C of temperature was found as the optimum for maximum cellulase production and this was reported(Ali *et al.,* 1991). He reported the maximum yield of cellulase from the fungus *Asapergillus terreus* at 40^{o}C using water hyacinth as substrate after 6 days incubation.

The cellulase production was found to be dependent upon the nature of the carbon source used in culture media. In the present study among the different carbon sources tested, cellulose promoted maximal enzyme yield when compared to others. This could be attributed to the rapid growth accomplished by the early availability of carbon source along with the substrates activity of cellulase, glucose. The earlier results also suggested that the supplementation of sugars with substrate was found to be most effective nutrient for promoting the activity of cellulase (Roche *et al.,* 1994).

The enzyme production is affected significantly by under different concentration of the organic nitrogen source and nitrogen level in the medium (Kim *et al.,* 2006 and Kim *et al.,* 2006). The result of the present study showed that the different sources have different effects on enzyme activity. Among the different nitrogen sources tested, the enzyme activity was higher with peptone. The similar result was reported (Mohapatra, 1997).

Cellulase production by Estuarine *Klebsiella ozeanae* was significantly enhanced by optimization of medium composition and culture conditions. A highest cellulase production of 4.01 U/ml/min was obtained in the medium. The economic medium composition and the culture conditions, the high cellulase activity, and the dominant occupation of cellulase in culture fluid enlighten the potential application of *Klebsiella ozeanae* for the production of cellulase. Given that cellulase is a natural product of bacteria in the marine environment, and that its presence enhances the bacterial use in ethanol production from cellulose.

References

Akiba, S., Y. Kimura, K. Yamamoto and Kumagai, H . 1995. Purification and characterization of a protease-resistant cellulase from Aspergillus niger. J. Ferment. Bioeng 79(2): 125-130.

Alani, F., W.A. Anderson andMoo-Young M. 2008.New isolate of Streptomyces sp. with novel thermoalkalotolerant cellulases. Biotechnol. Lett. 30:123-126.

Ali, S., A. Sayed, R.I. Sarkar and Alam, R. 1991. Factors affecting cellulase production by Aspergillus terreus using water hyacinth. J. Microbiol and Biotechno.l 7:62-66.

Bai, S., M. Ravi kumar, D.J. Mukesh kumar, P. Balashanmugam, M.D. Bala**,** M.D. kumaran, and Kalaichelvan, P.T. 2012. Cellulase Production by Bacillus subtilis isolated from Cow Dung. Arch of App Sci Resear. 4 (1):269-279.

Bai1, S., M. Ravi kumar, D.J. Mukesh kumar DJ, Balashanmugam P, Bala Coral G, Arikan B, Unaldi H.Guvenmes M. N.Some, 2002. properties of crude carboxymethyl cellulase of Aspergillus niger Z10 wild type strain. J. Biol ;26:209-213.

Castro, A.M., de Albuquerque M.L, de Carvalho A, Leie SGF and Pereira, N. 2010. Cellulase from Penicillium funiculosum: production, roperties and application to cellulose hydrolysis. J Ind Microbiol. Biotechnol. 37: 151–158.

Dashtban, M., M. Maki, K.T. Leung, C. Mao and Qin, W. 2010.Cellulase activities in biomass conversion: measurement methods and comparison. J. Biotechnol.30:302-309.

Ekperigin, M. M., 2007.Preliminary studies of cellulase production by Acinetobacter anitratus and Branhamella sp. Afr. J. Biotechno.6: 028-033.

Genansounou, E., 2010.Production and use of lignocellulosic bioethanol in Europe: current situation and perspectives. Bioresor. Technol . 101: 4842– 4850.

George, J, R. Arunachalam, K. Paulkumar, E.G. Wesely, S. Shiburaj and Annadurai G. 2010.Characterization and phylogenetic analysis of cellulase producing Streptomyces noboritoensis SPKC1. Interdiscip. Sci.2:205-212.

Ibarra, D., J.C.A. DelRio, A. Gutierrez , I.M. Rodriguez, J. Romero, M.J. Martinez and Martinez, A.T. 2004. Isolation of high-purity residual lignins from eucalypt paper pulps by cellulase and proteinase treatments followed by solvent extraction. Enz. Microbiol. Tecnol.35:173-181.

Kim, S. W., S.W. Kang and Lee , J. S. 1997. Cellulase and Xylanase Production by *Aspergillus niger* KKS in Various Bioreactors .J.Bioresou Tecnol. 59: 63-67.

Lee, S. J., Altaner, A. Puls J and Saake, B. Determination of the substituent distribution along cellulose acetate chains as revealed by enzymatic and chemical methods. Carbohy Poly 2003; 54: 353–36.

Lopez, A. M., Claassen, P. A. M. Mooibroek, A and, Vos W. M. D. 2000. Utilisation of saccharides in extruded domestic organic waste by Clostridium acetobutylicum ATCC 824 to produce acetone, butanol and ethanol. App. Microbiol. Biotechnol. 54:162–167.

Lynd, L.. R, Weimer P. J, Van W. H and Pretorius I .S. 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Mol .Biol . 66:506–577.

Maki, M., Leung KT, Qin W. 2009. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int. J. Biol. Sci . 5:500-516.

Mohapatra, B.R., 1997.Properties of carboxymethylcellulase (Endo- 1, 4-beta-gluconose) fro Bacillus sp isolated from the marine sponge (Axinella sp). Ind. J. Biotechnol;26:292-296.

Murashim,a K., Kosugi, A, Doi R. H. 2003.Solubilization of cellulosomal cellulases by fusion with cellulose-binding domain of noncellulosomal cellulose from Clostridium cellulovorans. Proteins; 50:620–628.

Nishiyama, Y., Sugiyama J. Chanzy H.Langan 2003.Crystal structure and hydrogen bonding system in cellulose I alpha from synchrotron X-ray and neutron fiber diffraction. J. Am Chem Soc.125:14300–14306.

Pason, P., Kosugi A, Waeonukul R, Tachaapaikoon C, Ratanakhanokchai

K, Arai T, Murata Y, Nakajima J, Mori Y. 2010.Purification and characterization of a multienzyme complex produced by Paenibacillus curdlanolyticus B-6. App. Microbiol. Biotechnol .85:573-580.

Pothiraj C, Balaji P, Eyini M. 2006. Enhanced production of cellulase by various fungal cultures in solid state fermentation of Cassava waste. Afr. J. Biotechnol. 5:1882-1885.

Prasad, M.P., and R.Sethi, (2013). Optimization of cellulase production from a novel bacterial isolate mesorhizobium sp. From marine source. J.Enz. Res. 4:39-45.

Praveen, B., Viswanath, K. Usha, K.Y. Pallavi, H.. Reddy G.V.S, Naveen, M. Reddy B.R, 2011.Lignolytic enzymes of a mushroom – Stereum ostrea isolated from wood logs. Enz. Res. 1:1-6.

QIN, Y., He, H.; Li, N.; Ling, M. and Liang, Z. 2010. Isolation and characterization of a thermostable cellulase-producing Fusarium chlamydosporum. J.Microbiol and Biotechnol, 26 (11): 1991-1997.

Roche N, Desgragnes C, Durand A. 1994. Study on the solid-state production of a thermostable a-L-arabinofura-nosidase of Thermoascus aurantiacus on sugar beet pulp. J. Biotechnol. 38: 43-50.

Schwarz, W. H., 2001.The cellulosome and cellulose degradation by anaerobic bacteria. App. Microbiol, Biotechnol; 56**:**634–649.

Sing, A., and Hayashi K. 1995. Microbial cellulase. Protein Architecture Molecular properties and Biosynthesis. Adv. Appl. Microbial 40:1-35.

Verma, V., A. Verma and Kushwaha, A. 2012. Isolation & production of cellulase enzyme from bacteria isolated from agricultural fields in district Hardoi, Uttar Pradesh, India. Adv . App. Sc.i Res. (1):171-174.

Waeonukul, R., K.L. Kyu, K. Sakka and Ratanakhanokchai, K. 2009. Isolation and characterization of a multienzyme complex (cellulosome) of the *Paenibacillus curdlanolyticus* B-6 grown on Avicel under aerobic conditions. J. Biosci. Bioeng. 107:610-614.

Wang, C., Y, Hsieha Y. R, Nga C. C, Chanb H, Linc H, Tzenga W. S and Shyua Y. T. 2009.Purification and characterization of a novel halostable cellulase from Salinivibrio sp. strain NTU-05. J. Microbiol, Biotechnol.7: 311-315.

Xiaoting, C., C. Fangfang, C. Liqun, Z. lin, L. Guodong and Zonghua, W. 2008.Isolation and Anaiysis of Oxalic Acid-insenstive Mutant of Arabidopsis. Thaliana.J.biotecnol: 203-208.