American Journal of Microbiology 5 (1): 18-26, 2014
ISSN: 1948-982x

doi:10.3844/ajmsp.2014.18.26 Published Online 5 (1) 2014 (http://www.thescipub.com/ajm.toc)

TESTING CARBOXYMETHYL CELLULASE ACTIVITY SECRETED BY TRICHOPHYTON TERRESTRE IN CARBOXYMETHYL CELLULOSE SOLUTION

Mansoor Ahmad Lone, Sanjay Sahay, Ravinder and Deepak

Department of Botany, Govt. Science and Commerce College, Benazeer, Bhopal-462 008 (MP), India

Received 2013-11-22; Revised 2013-12-17; Accepted 2014-08-12

ABSTRACT

The techniques employed for bioconversion of cellulosic biomass to simple sugars by cellulases have a great industrial brunt. Cold-adapted microorganisms are potential resource of cold-active carboxymethyl cellulases secluded from the cold regions. In this study, *Trichophyton terrestre*-a rare fungal species in Indian soils, isolated from the rhizosphere of *Juglans regia* L. during winter season was subjugated for the production and commotion of an enzyme carboxymethyl cellulase in carboxymethyl cellulose solution by DNS method at an ample range of temperatures, using Lineweaver-Burk plot which offers a practical graphical method for the analysis of Michealis-Menten equation, to establish the imperative terms in enzyme kinetics as K_m and V_{max}. The enzyme kinetic parameters like maximum activity (V_{max}), K_m and turnover number were recorded at varying concentrations of CMC and different temperatures (4°C and 50°C). The enzyme was found to be tolerant and stable at two varying temperatures which enables this fungal species to survive in the extreme environmental conditions of northern India. Such property of carboxymethyl cellulase enzyme has extensive range of applications and the potential to open new application fields in the areas of industrial processes.

Keywords: Carboxymethyl Cellulase, *Trichophyton Terrestre*, CMC, Glucose, Enzyme Kinetics

1. INTRODUCTION

Human kind has benefited enormously from the study of microbes since their discovery in the 17th century. However, such benefits have come from the study of just a miniscule fraction of the millions of the species of microbes in the environment (Anne and Ann, 2007). The enormous returns have been particularly derived from those microbes which are able to live in the severe environmental conditions like low or high temperature, extremes of pH conditions, high salt concentrations, nutrient deficient soils. The tendency of surviving in such environmental conditions comes more or less directly from the enzyme stability which enhances the survival rates of these microbes in harsh conditions. Sometimes these microbes are called as the natures 'master chemists' because an endless variety of chemical compounds are derived from their community. They produce the novel and stable enzymes which function under extreme conditions comparable to those prevailing in various industrial processes. Keeping in mind that intense environments always provide a unique resource of microorganisms and novel biocatalysts, *Trichopyton terrestre* was isolated from the rhizosphere of *Juglans regia* L. in the Kashmir valley of India which has its unique environment throughout the world.

Cellulose is the principal structural polysaccharide in plants and the most abundant biomass on earth. It is composed of β-1, 4-linked glucose units which contains both highly crystalline and amorphous regions (Zhang and Lynd, 2004). Researchers have

Corresponding Author: Mansoor Ahmad Lone, Department of Botany, Govt. Science and Commerce College, Benazeer, Bhopal-462 008 (MP), India Tel: +91 9797025562

been stimulated to hydrolyze cellulose to soluble sugars by microorganisms for industrial processes owing to great potentiality of this abundant natural product as an alternative energy source (Coughlan, 1990). Cellulosic materials are converted into soluble sugars by many methods like acid hydrolysis, pyrolysis and by employing enzyme cellulase (Cooney *et al*., 1978). The acid hydrolysis of cellulosic materials is cheaper than the cellulase hydrolysis, but the former often requires high temperature, pressure and leads to the accumulation of abhorrent by-products (Fennington *et al*., 1982). On the other hand, enzymatic hydrolysis does not have these tribulations. Mostly bioconversion of cellulose by enzymes is employed in the industrial processes to synthesize commercially important products. Cellulase refers to the family of enzymes that work to hydrolyze cellulose. There are numerous microorganisms including both bacteria and fungi which have been found to produce a variety of cellulases for the degradation of cellulose (Miranda *et al*., 2011) but only a restricted group is capable to produce a sufficient amount of cell-free cellulase, which is proficient in completely hydrolyzing the crystalline cellulose in vitro. For the degradation of cellulose, fungi utilize the hydrolytic enzymes viz., exo-cellulase, endo-cellulase, cellobiohydrolase, endogluconase and β-glycosidase (Bhat, 2000). Continuing research on *Trichophyton terrestre* indicated that the microorganism has a complete set of cellulase enzymes required for the breakdown of cellulose to glucose (Reese *et al*., 1950). Cellulase enzymes indicate the potentiality of successful conversions of waste cellulosics into foods for our burgeoning population, thus the enzyme exploitation subject demands the intense research even at molecular level (Demain and Solomon, 1985).

2. MATERIALS AND METHODS

2.1. Isolation and Identification

Soil samples were collected in aseptic manner roughly 20 cms deep from the rhizosphere of *Juglans regia* L. in the Northern regions of India (Kashmir valley) during three different seasons-rainy, spring and winter. Fungal colonies were isolated by serial dilution method to get more manageable results (Aneja, 2003). 1 g of soil was transferred to 10 mL of distilled water in test tubes. Dilutions were made up to 10^{-6} and fungal culturing was done by using Czapek-Dox agar medium with following composition (g/L); sucrose- 30, $NaNO_3$-3, K_2HPO_4-1, $MgSO_4$-0.5, KCl-0.5, $FeSO_4$-0.01, agar agar-15, pH of the medium was adjusted to 7.3. After autoclaving at 121°C and 15 lbs pressure, 20 mL of sterile medium was transferred to sterile petri-plates and allowed for solidification (Chloramphenicol 250 mg/100 mL was also added to check the bacterial growth). After solidification of the medium, 0.1 mL of soil suspension was spread with the help of spreader and incubated at 28°C for 7 days. The fungal cultures grown on the medium were transferred on to the Potato Dextrose Agar (Hi Media) medium and pH was maintained at 5.6 for further studies. Identification of fungi was done based on cultural, morphological and microscopic characters.

2.2. Cellulase Production

Among the identified cultures, Trichophyton terrestre, a fungal species rarely present in Indian soils, was selected to know its potential for carboxymethyl cellulase production and activities. A volume of 100 mL of Czapek-Dox broth medium amended with 1% cellulose was distributed into separate 250 mL Erlenmeyer conical flask. The pH of the medium was adjusted to 5. After autoclaving at 121°C and 15 lb pressure, the fungal spore suspension was inoculated into the conical flask with the inoculum concentration of 1×106 spores mL^{-1}. The flask was incubated at 32°C on a rotary shaker at 120 rpm for 7 days (Lone *et al*., 2012). After incubation, the content of the flask was passed through Whatman filter paper No.1 to separate the mycelial mat from culture filtrate. The filtrate thus obtained was used for the estimation of extracellular protein content and total activity of cellulases.

2.3. Protein Estimation

Protein content of the supernatant secreted by fungus was estimated by Lowry's method (Lowry *et al*., 1951). The optical density of the strain-supernatant was compared with the BSA standard curve (**Fig. 1**) to calculate the amount of protein (mg/mL) present in the supernatant used in cellulase assay.

Preparation of Glucose standard curve.

Glucose stock: 100 mg of glucose was dissolved in 10 mL distilled water (10 mg mL^{-1}).

Citrate buffer: 210 g citric acid monohydrate $C_6H_{80}.7H_2O$ was dissolved in 750 mL distilled water. NaOH was added until pH equals to 4.3 and final make up was 1000 mL. This is 1 M citrate buffer having pH of 4.5, when it was diluted to 0.05, pH was maintained at 4.8 (0.05 M Citrate buffer pH 4.8).

Dinitrosalicylic Acid (DNS): 30 g K-Na tartarate, dissolved in 50 mL of distilled water was further added by 1 g DNS and 20 mL 2N NaOH and final make up was 100 mL.

The experimental data was collected and glucose standard curve (**Fig. 2**) was used as a standard (Kondo *et al*., 1994).

Kinetics of enzyme cellulase: Carboxymethyl cellulase in the crude extract was assayed with increasing concentrations of carboxymethyl cellulose (0.16-0.83 mg/0.5 mL) at 4°C and 50°C at pH 6.5. Enzyme catalyzed reactions often exhibit a special form of kinetics, called Michealis Menten kinetics, which are characterized by hyperbolic relationship between reaction Velocity (V) and substrate concentration (S). Normal enzyme kinetic values are measured under steady-state conditions and such conditions usually prevail in the cell. For many enzyme-catalyzed reactions, the kinetics under steady-state can be described by a simple expression known as Michealis Menten kinetics:

$$V = V_{max} S / K_m + S$$

Where:

V = The observed rate or velocity
V_{max} = The maximum velocity (at infinite substrate concentration)
S = The substrate concentration and
K_m = The Michaelis-Menten constant which represents the concentration of substrate required to half saturate the enzyme

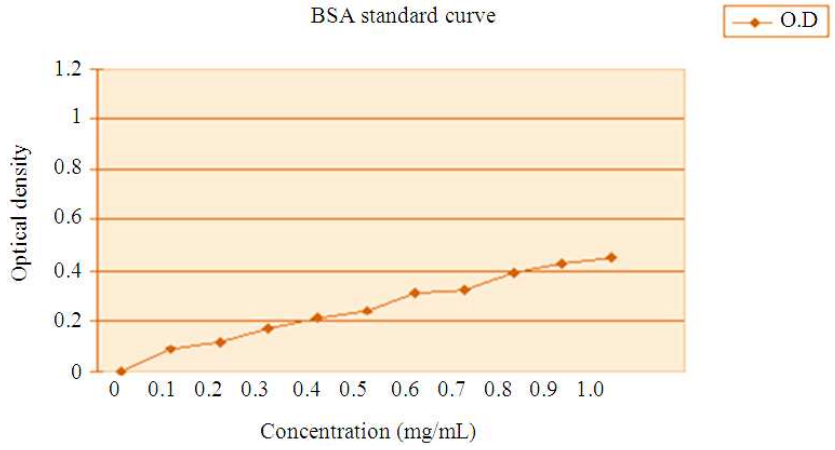

Fig. 1. BSA standard curve

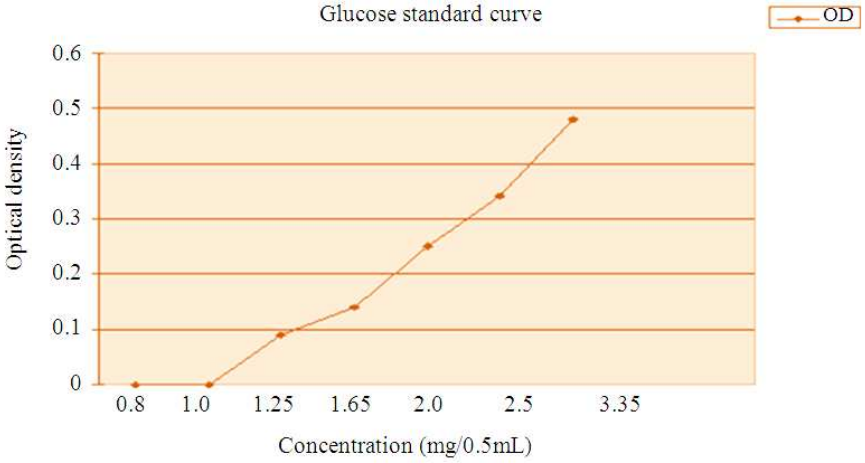

Fig. 2. Glucose standard curve

The Lineweaver-Burk plot was used to determine the important kinetics parameters of carboxymethyl cellulase enzyme such as K_m and V_{max} (Lineweaver and Burk, 1934). The computerized curve was obtained by fitting the values 1/S on x-axis and $1/V_{max}$ on y-axis. The y-intercept of such a graph denotes $1/V_{max}$, while the x-intercept represent-$1/K_m$. The turnover number, the number of substrate molecules converted into products per unit time per molecule of enzyme, was also obtained by dividing the values of V_{max} by the amount of enzyme used in the experiment. Typical turnover number values ranges from 10^2 to 10^3 S-1.

3. RESULTS

Among an array of fungal strains isolated during rainy, spring and winter seasons from the rhizosphere of *Juglans regia* L., geophilic fungus *Trichophyton terrestre* was isolated only during the winter season and screened for the production and activity of the enzyme carboxymethyl cellulase. The enzyme secreted by the fungal species in culture solution at 32°C was purified to homogeneity. All enzymes that act upon the cellulose are deemed to move about the surface in a random walk (Nimlos *et al*., 2007; Ting *et al*., 2009) in steps equating to the dimensions of one glucose molecule (5 Å) per time-step.

The activity of the enzyme was assayed in the carboxymethyl cellulose solution with the increasing substrate concentrations. Glucose production by enzyme participation was determined at 4°C and 50°C by DNS method thereby generating a reddish brown colour for amino compounds (Vancov and Keen, 2009). The glucose liberation in enzymatic reaction with the chromogenic agents occurs chromogenically in the reaction. The absorbance was measured by the spectrophotometeric method at the wavelength of around 540 nm (**Fig. 3**) (Coleman *et al*., 2007). Enzyme activity with 0.83 mg/0.5mL CMC concentration was found to be 52.5 µmoL/mg protein and 69.37 µmol/mg protein at 4°C and 50°C respectively (**Fig. 4**). The enzyme remained active at two different temperatures. A broad temperature optimum was observed for the enzyme carboxymethyl cellulase at 4°C and 50°C. However, the enzyme has been found to be active at 100°C (Nataraja *et al*., 2010). The turnover number was observed at varied CMC concentrations. The maximum turnover number at 4°C and 50°C was found to be 105 mol/0.5mL enzyme/30 min and 138.74 mol/0.5mL enzyme/30 min respectively (**Fig. 5**), when the concentration of carboxymethyl cellulose was 0.83 mg/0.5mL.

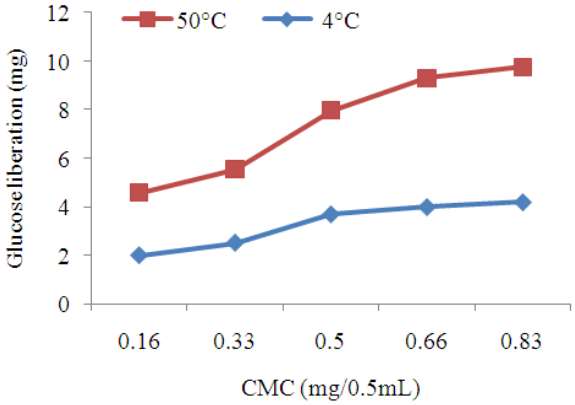

Fig. 3. Glucose liberation (IU/mg protein) at 4°C and 50°C with increasing concentration of CMC (mg/0.5mL)

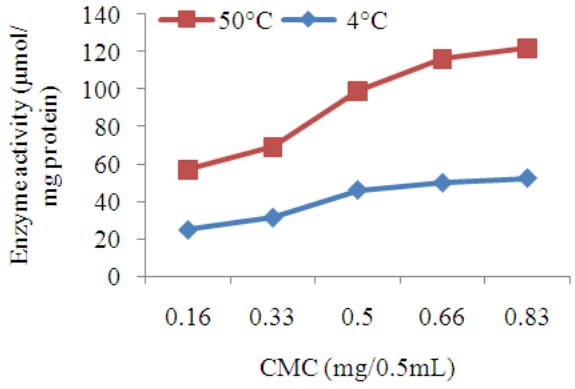

Fig. 4. Enzyme activity (µmol/mg protein) at 4°C and 50°C with increasing concentration of CMC (mg/0.5mL)

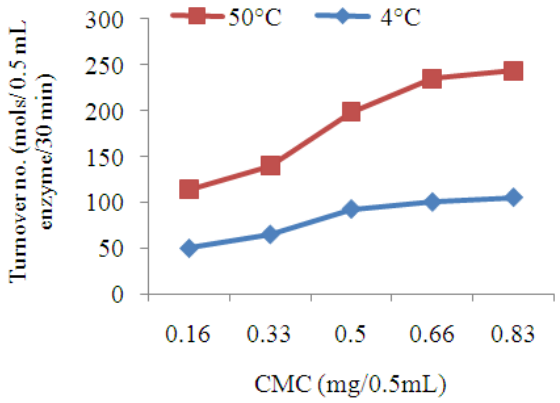

Fig. 5. Turnover number (mols/ 0.5 ml enzyme/30 min) at 4°C and 50°C with increasing concentration of CMC (mg/0.5mL)

The activities of carboxymethyl cellulase from *Trichophyton terrestre* were greatly influenced by the concentration of the substrate. During the initial stage of hydrolysis cellulose released more amount of glucose to the solution (Andrew *et al*., 2011). It is perceptible that the concerned mechanisms are extremely multifaceted, since the enzyme effect coupled with intrinsic properties of substrate such as degree of polymerization, crystallinity, or accessible surface area which play crest roles in the enzymatic degradation.

The most important finding was the extraordinary level of tolerance at low and higher temperatures along with a great turn over number. The enzymes showing activity at wide range of temperatures are today the enzymes of choice for microbiologist, biochemists, biotechnologists and industrialists (Kazem, 2012). Cellulases active at low and high temperatures are promising enzymes to reinstate the conventional enzyme processes. There is an immediate need for the selection of ideal cellulases to retain the activity at extreme range of temperatures which may be utilized in a variety of fields in order to obtain the best results. In this direction, *Trichophyton terrestre* could be used as a prospective source of enzyme cellulases. Extensive research is vital to unknot the full potential of such micro-organisms.

Being the fingerprint of an enzyme, K_m value is considered the most important criterion to evaluate the enzyme for various uses. Its value indicates the higher efficiency of the enzyme, lesser the K_m value, higher the efficiency of an enzyme. The K_m value of enzyme carboxymethyl cellulase determined at 4°C and 50°C was found to be 0.312mM and 0.67mM respectively (**Fig. 6 and 7**).

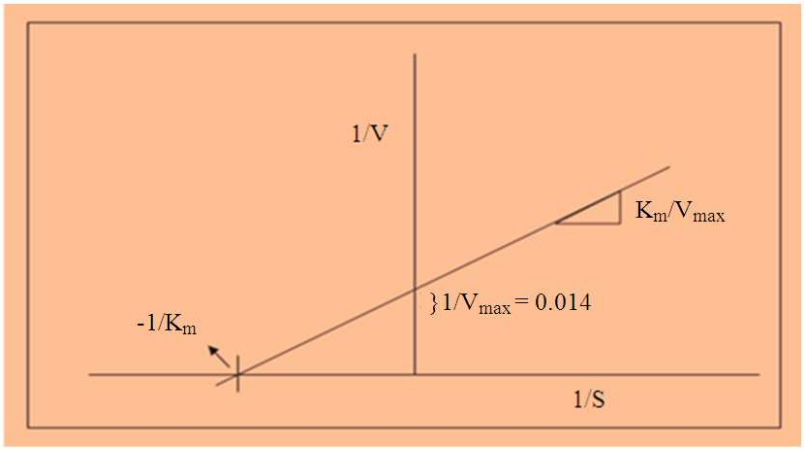

Fig. 6. Km determination at 4°C

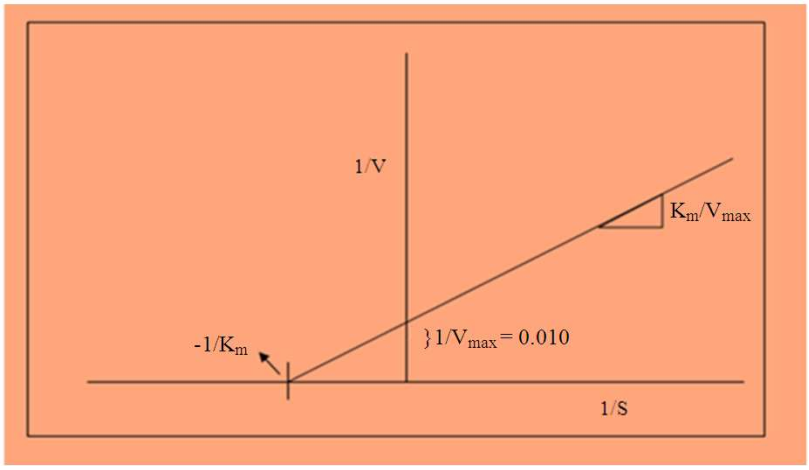

Fig. 7. K_m determination at 50°C

The enzyme carboxymethyl cellulase remained more active at 4°C temperature when the concentration of CMC was 0.83 mg/0.5 mL. The biocatalysts which remain active at cold conditions find their place in chemical synthesis and transformation, bioremediation of contaminants and clean-energy production, confirming and reinforcing the potential of this technology for environmental purposes. Intercept or $1/V_{max}$ at 4°C and 50°C calculated as 0.014 min/mM and 0.010 min/mM respectively. The slope (K_m/V_{max}) was found to be 0.0043 mM/min and 0.0067 mM/min at 4°C and 50°C respectively. The maximum activity (V_{max}) determined at 4°C and 50°C was 71.42 µmol/mg protein and 100 µmol/mg protein respectively. Industrial enzymes working at different temperatures allow technologists to develop processes which closely approach the gentle and efficient processes in nature.

4. DISCUSSION

The determination of the kinetic parameters for CMC hydrolysis reveals a fascinating phenomenon of kinetic optimization at different temperatures (Tong *et al*., 1980). The activity of the enzyme carboxymethyl cellulase at low and high temperature can be viewed as the main physiological adaptation to cold and high temperatures at the enzyme level, as it compensates for the reduction of chemical reaction rates induced by low and high temperatures (Feller and Gerday, 2003; Carrard *et al*., 2000). Because of the extreme molecular stability of cellulases at wide array of temperatures, *Trichophyton terrestre* form an interesting enzyme source for industrial applications and are used in textile industries (Gusakov *et al*., 2000; Belghith *et al*., 2001), in detergents (Maurer, 1997), pulp and paper industry (Buchert *et al*., 1996), in food industry (Galante *et al*., 1998), besides account for a significant share of the world enzyme market. In addition to the above applications, cellulases are also employed in generation of antibacterial chito-oligosaccharides which may well be used in food preservation (Liu and Zhu, 2000), immuno-modulation (Tsai *et al*., 2000) and potent antitumor agents (Wu and Tsai, 2004).

In the biocatalyzed-reaction networks, inhibitory effects of the reaction intermediates play an important role in determining the enzymatic kinetics. The intrinsic reaction kinetics of enzymatic cellulose hydrolysis is also subjected to mediation by a host of factors like inhibitory effects of reaction intermediates and enzyme adsorption. The soluble products such as cellobiose and glucose have been reported to be the inhibitors of the cellulase complex (Howell and Stuck, 1975; Katz and Reese, 1968; Ghose, 1977; Nisizawa, 1973), individual enzyme endoglucanase components (Halliwell and Griffin, 1973; Ladisch *et al*., 1980), cellobiohydrolase (Hsu *et al*., 1980) and β-glucosidase (Wood and Mc Care, 1975; Gong *et al*., 1997). In recent years, the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Akpan *et al*., 1999; Abu *et al*., 2005).

The optimal pH of 6.5 for the cellulase activity is in conformity to that of (Catriona *et al*., 1994), who reported a broad pH range of 5.0 to 7.0 over which the cellulases were highly active. Bok *et al*. (1998) also reported a pH range of 6.0 to 6.6 for two thermostable endocellulases from Thermotoga neapolitana. The physicochemical or ecological conditions existing during the enzyme action need to be taken into account to gain a better understanding of any experimental observation. Specifically, the substrate composition, crystallinity and recalcitrance (Fierobe *et al*., 2002; Himmel *et al*., 2007; Jeoh *et al*., 2006) have a distinctive influence on the mechanism and dynamics of cellulose strap and hydrolysis (Woodward *et al*., 1992; Beguin and Aubert, 1994).

5. CONCLUSION

The present study, confirmed that the enzyme carboxymethyl cellulase from *Trichopyton terrestre* can tolerate different temperatures which enables this fungal species to survive in the extreme environmental conditions of northern India, where the winter temperature goes down to -20°C and touches the upper limit of 37°C in the summer season. Such a great fluctuation in the temperature occurs only in few regions of India which could be the reasonable factor for the restricted distribution of *Trichophyton terrestre* in Indian soils. The biocatalysts which remain active at cold and hot conditions are desirable in many industrial processes. However, an extensive research work is required to overcome several bottlenecks such as less explored biodiversity of psychrophilic and thermophilic microbes, low activity and stability in terms of turnover number of enzymes under varied environmental conditions.

6. ACKNOWLEDGEMENT

The authors are gratified to the Principal, Govt. Science and Commerce College Benazeer, Bhopal-462

008 (MP), India and for providing all the requisite infrastructural support and laboratory facilities with regard to complete these experiments which were purely funded by authors.

6.1. Author's Contributions

All authors equally contributed in this work.

6.2. Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

7. REFERENCES

Abu, E.A., S.A. Ado and D.B. James, 2005. Raw starch degrading amylase production by mixed culture of Aspergillus Niger and Saccharomyces Cerevisae grown on Sorghum pomace. Afr. J. Biotechnol., 4: 785-790.

Akpan, I., M.O. Bankole, A.M. Adesemowo and G.O. Latunde-Dada 1999. Production of amylase by A. niger in a cheap solid medium using rice band and agricultural materials. Trop. Sci., 39: 77-79.

Andrew, C.W., A.L. Bryce and S.H. Victoria, 2011. A cellular automaton model of crystalline cellulose hydrolysis by cellulases. Biotechnol Biofuels., 4: 39-39. PMID: 22005054

Aneja, K.R., 2003. Experiments in Microbiology, Plant Pathology And Biotechnology. 4th Edn., New Age International, New Delhi, ISBN-10: 812241494X. pp: 607.

Anne, J. and R. Ann, 2007. The New Science of Metagenomics: Revealing the Secrets of our Microbial Planet. 1st Edn., National Academies Press, ISBN-10:0309185750. pp: 148.

Beguin, P. and J. Aubert, 1994. The biological degradation of cellulose. FEMS Microbiol., 13: 25-58. DOI: 10.1016/0168-6445(94)90099-X

Belghith, H., C.S. Ellouz and A. Gargouri, 2001. Biostoning of denims by *Penicillium occitanis* (Pol6) Cellulases. J. Biotechnol., 89: 257-262. DOI: 10.1016/S0168-1656(01)00309-1

Bhat, M.K., 2000. Cellulases and related enzymes in biotechnology. Biotechnol. Adv., 18: 355-383. DOI: 10.1016/S0734-9750(00)00041-0

Bok, J.D., D.A. Yernool and D.E. Eveleigh, 1998. Purification, characterization and molecular analysis of thermostable cellulases celA and celB from Thermotoga neapolitana. Appl. Environ. Microbiol., 6: 4774-4781. PMID: 9835561

Buchert, J., A. Suurnakki, M. Tenkanen and L. Viikari, 1996. Enzymatic Characterization of Pulps, In: Enzymes for Pulp and Paper Processing, Jeffries, T.W. and L. Viikari (Eds.)., American Chemical Society, Washington, ISBN-10: 0841234787, pp: 38-43.

Carrard, G., A. Koivula, H. Soderlund and P. Beguin, 2000. Cellulose-binding domains promote hydrolysis of different sites on crystalline cellulose. Proc. Natl. Acad. Sci. U.S.A., 97: 10342-10347. PMID: 10962023

Catriona, A.W., I.M. Sheila and M.W. Thomas, 1994. Characterization of a β-D-glucosidase from the anaerobic rumen fungus Neocallimastix rontalis with particular reference to attack on cello-oligosaccharides. J. Biotechnol., 37: 217-227. DOI: 10.1016/0168-1656(94)90129-5

Coleman, D.J., M.J. Studler and J.J. Naleway, 2007. A long-wavelength fluorescent substrate for continuous fluorometric determination of cellulase activity: Resorufin-β-D-cellobioside. Anal. Biochem., 371: 146-153. DOI: 10.1016/j.ab.2007.08.027

Cooney, C.L., D.I.C. Wang, S.D. Wang, J. Gordon and M. Jiminez, 1978. Simultaneous cellulose hydrolysis and ethanol production by a cellulolytic anaerobic bacterium. Biotechnol. Bioeng., 8: 103-114.

Coughlan, M.P., 1990. Cellulose Degradation by Fungi. In: Microb. Enz. biotechnol, Fogarty WM, Kelly CT (Eds.)., United Kingdom, London, pp: 1-36.

Demain, A.L. and N.A. Solomon, 1985. Biology of Industrial Microorganisms. Ist Edn., Benjamin/Cummings, Menlo Park, ISBN-10: 0805324518. pp: 573.

Feller, G. and C. Gerday, 2003. Psychrophilic enzymes: Hot topics in cold adaptation. Nat. Rev. Microbiol., 1: 200-208. PMID: 15035024

Fennington, G., D. Lupo and F. Stutzenberger, 1982. Enhanced cellulase production in mutants of Thermomonospora curvata. Biotechnol. Bioeng., 24: 2487-2497. PMID: 18546218

Fierobe, H., E. Bayer, C. Tardif, M. Czjzek and A. Mechaly *et al*., 2002. Degradation of cellulose substrates by cellulosome chimeras. Substrate targeting versus proximity of enzyme components. J. Biol. Chem., 277: 49621-49630. PMID: 12397074

Galante, Y.M., D. Conti and R. Monteverdi, 1998. Application of Trichoderma Enzymes in Food and Feed Industries. In: Trichoderma and Gliocladium: Enzymes, Biological Control and Commercial Applications, Harman, G.F. and C.P. Kubicek, (Eds.)., Taylor and Francis Limited, London, ISBN-10: 0748408053. pp: 2327-342.

Ghose, T.K., 1977. Cellulase biosynthesis and hydrolysis of cellulosic substances. Adv. Biochem. Eng., 6: 39-76. DOI: 10.1007/3-540-08363-4_2

Gong, C.S., M.R. Ladisch and G.T. Tsao, 1997. Cellobiase from Trichoderma viride: Purification, properties, kinetics and mechanism. Biotechnol. Bioeng., 19: 959-81. PMID: 560223

Gusakov, A.V., A.G. Berlin, A.N. Popova, O.N. Okunew and A.P. Sinitsyna, 2000. A comparative study of different cellulase preparations in the enzymatic treatment of cotton fabrics. Apple. Biochem. Biotechnol., 88: 119-126. DOI: 10.1385/ABAB:88:1-3:119

Halliwell, G. and M. Griffin, 1973. The nature and mode of action of the cellulolytic component C1 of Trichoderma koningii on native cellulose. Biochem, J., 135: 587-594. PMID: 4798312

Himmel, M., S. Ding, D. Johnson, W. Adney and M. Nimlos *et al*., 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science, 315: 804-807. DOI: 10.1126/science.1137016

Howell, J.A. and J.D. Stuck, 1975. Kinetics of solka floc cellulose hydrolysis by trichoderma viride cellulase. Biotechnol. Bioeng., 17: 873-893. DOI: 10.1002/bit.260170608

Hsu, T.A., C.S. Gong and G.T. Tsao, 1980. Kinetic studies of cellodextrins hydrolyses by exocellulase from trichoderma reesei. Biotechnol. Bioeng., 22: 2305-2320. DOI: 10.1002/bit.260221108

Jeoh, T., D. Wilson and L. Walker, 2006. Effect of cellulase mole fraction and cellulose recalcitrance on synergism in cellulose hydrolysis and binding. Biotechnol. Prog., 22: 270-277. PMID: 16454519

Katz, M. and E.T. Reese, 1968. Production of glucose by enzymatic hydrolysis of cellulose. Applied Microbiol., 16: 419-420.

Kazem, K., 2012. Hyperthermophiles: Metabolic Diversity and Biotechnological Applications. In: Extremophiles: Microbiology and Biotechnology, Anitori, R.P. (Ed.)., Horizon Scientific Press, Norfolk, ISBN-10: 1904455980, pp: 206-206.

Kondo, A., T. Urabe and K. Higashitani, 1994. Bioconversions in aqueous two-phase system using enzymes immobilized on ultrane silica particles. J. Ferment. Bioeng., 77: 700-703.

Ladisch, M.R., C.S. Gong and G.T. Tsao, 1980. Cellobiose hydrolysis by endoglucanase (glucan glucanhydrolase) from trichoderma reesie: Kinetics and mechanism. Biotechnol. Bioeng., 22: 1107-1126. DOI: 10.1002/bit.260220602

Lineweaver, H. and D. Burk, 1934. The determination of enzyme dissociation constants. J. Am. Chem, Soc., 56: 658-666. DOI: 10.1021/ja01318a036

Liu, W. and W.M. Zhu, 2000. Production and regeneration of Trichosporon cutaneum protoplasts. Process Biochem., 35: 659-664. DOI: 10.1016/S0032-9592(99)00115-6

Lone, M.A., M.R. Wani, N.A. Bhat, S.A. Sheikh and M.A. Reshi, 2012. Evaluation of cellulase enzyme secreted by some common and stirring rhizosphere fungi of *Juglans regia* L. by DNS method. J. Enz. Res., 3: 18-22.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-75. PMID: 14907713

Maurer, K.H., 1997. Development of New Cellulases, In: Enzymes in Detergency, Jan, H.V.E. and M. Onno, (Eds.)., Taylor and Francis, New York, ISBN-10: 082479995X. pp: 175-202.

Miranda, L.M., B. Michael, T.L. Kam and Q. Wensheng, 2011. Characterization of some efficient cellulase producing bacteria isolated from paper mill sludges and organic fertilizers. Int. J. Biochem. Mol. Biol., 2: 146-154. PMID: 21969070

Nataraja, S., D.M. Chetan and M. Krishnappa, 2010. Effect of temperature on cellulose enzyme activity in crude extracts isolated from solid wastes microbes. Intl. J. Microbiol. Res., 2: 44-47.

Nimlos, M.R., J.F. Matthews, M.F. Crowley, R.C. Walker and G. Chukkapalli *et al*., 2007. Molecular modeling suggests induced fit of Family I carbohydrate-binding modules with a broken-chain cellulose surface. Prot. Eng. Des. Sel., 20: 179-187. PMID: 17430975

Nisizawa, K., 1973. Mode of action of cellulases. J. Ferment. Technol., 51: 267-304.

Reese, E.T., R.G.H. Sui and H.S. Levinson, 1950. The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol., 59: 485-497. PMID: 15436422

Ting, C.L., D.E. Makarov and Z.G. Wang, 2009. A kinetic model for the enzymatic action of cellulase. J. physc. Chem. B., 113: 4970-4977. PMID: 19292431

Tong, C.C., A.L. Cole and M.G. Shepherd, 1980. Purification and properties of the cellulases from the thermophilic fungus Thermoascus aurantiacus. Biochem. J., 191: 83-94. PMID: 6781492

Tsai, G.J., Z.Y. Wu and W.H. Su, 2000. Antibacterial activity of a chitooligosaccharide mixture prepared by cellulase digestion of shrimp chitosan and its application to milk preservation. J. Food Prot., 63: 747-752. PMID: 10852568

Vancov, T. and B. Keen, 2009. Rapid isolation and high-throughput determination of cellulase and laminarinase activity in soils. J. Microbiol. Methods, 79: 174-177. DOI: 10.1016/j.mimet.2009.08.013

Wood, T.M. and S.T. Mc Care, 1975. Symposium on Enzymatic Hydrolysis of Cellulose. 1st Edn., Finnish National Fund for Research and Development, Helsinki, ISBN-10: 9519250174. pp: 525.

Woodward, J., K.A. Affholter, K.K. Noles, N.T. Troy and S.F. Gaslightwala, 1992. Does cellobiohydrolase II core protein from Trichoderma reesei disperse cellulose macrofibrils?. Enz. Microb. Technol., 14: 625-630. DOI: 10.1016/0141-0229(92)90037-O

Wu, G.J. and G.J. Tsai, 2004. Cellulase degradation of shrimp chitosan for the preparation of water-soluble hydrolysate with immmunoactivity. Fisheries Sci., 70: 1113-1113.

Zhang, Y.H.P. and L.R. Lynd, 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. Biotechnol. Bioeng., 88: 797-824. PMID: 15538721