Optimization of cellulose production by *Bacillus subtilis* and its application in biomass saccharification

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Abstract

Cellulase has a wide range of applications in a variety of sectors. Keeping in view these facts about cellulase and its usability in industrial sectors, the present study was focused to optimise cultural condition for maximum cellulase production on different parameters. Out of 29 *Bacillus subtilis* exhibited maximum enzymatic activity in primary as well secondary screening. The organism identified on the basis of morphological, physiological and biochemical basis as mentioned in Bergey's manual. Maximum endoglucanase (EG) enzyme and amount of reducing sugar (RS) observed at 48 hours, 37±2°C and pH 7.0 when culture medium inoculated with 2% inoculum. Enzymatic activity increased in the presence of Maltose, dextrose, lactose, sucrose andPeptone was found as a best nitrogen source. Among different lignocellulosic materials Rice straw was the best substrate for production of enzyme and reducing sugar when *B. subtilis* use separately as well in consortia.

Keyword : Cellulose, lignocellulose, cellulase, *Bacillus subtilis*.

INTRODUCTION

Cellulose is the most abundant biomass and most dominatingagricultural waste on earth (Tomme*et al*., 1995). It is a polymer chain of glucoseunits connected by β-1, 4 linkages. Cellulose waste is ahuge renewable bioresource produced by the photosyntheticprocess (Jarvis 2003; Zhang and Lynd 2004). It has a high potential for bioconversion toimportant bioproducts such as ethanol. The ability to obtaincheap ethanol will depend on the successful identification ofnovel cellulase producing strains (Lee *et al*., 2008).

Microorganisms thatcan produce cellulase enzymescan degrade cellulose. These enzymes are commonly produced by some bacterialgenera such as *Cellulomonas*, *Pseudomonas*(Nakamura and Kppamura 1982)*Bacillus*, and*Micrococcus*(Immanuel*et al*., 2006)and fungi (Shin*et al*., 2000)that are widely used now inindustrial applications. Cellulosic biomass hydrolysis requiressuccessive action of three types of enzymes, which are cellobiohydrolase,endoglucanasecarboxymethyl cellulase (CMCase),and β-glucosidases(Bhat 2000).

Scientific research efforts try to improve the hydrolysis processin an economical way. Various parameterslike media components (carbon, nitrogen, mineralsources, medium additives and presence of inducers) andphysical parameters (pH, temperature) can be controlledto improve enzyme productivity and yield. Thesefactors are really important and highly influential in theenzyme production cost which is commonly considered asthe major bottleneck of the biotechnological processes (Lynd*et al*., 2002).

In natural, deconstruction process of lignocellulosicbiomass or plant biomass is corporative functions of bacterial and fungalspecies that accelerate the breakdown of substrates (Xu*et al*., 2013). Therefore,searching for cellulase produced from bacteria has possibility todiscover interesting enzyme properties that may be applied various. This study focused on optimize the nutritional and environmental parameters for improving cellulase production by bacterial strains.

MATERIALS AND METHODS

Identification of cellulolytic microbes

On the basis of their morphological, physiological and biochemical characteristics following Bergey's Manual of determinative bacteriology and methods given by Cappuccino and Sherman (1993) the cellulolytic bacteria was identified to be *Bacillus subtilIs.*

Enzyme Production medium and Submerged fermentation process

The fermentation media containing (g/L):yeast extract 0.5; peptone 3.0; $(NH_4)SO_4$ 1.5; K_2HPO_4 3.0; KH_2PO_4 4.0; $MgSO_4.7H_2O$ 0.3; $CaCl_2.7H_2O$ 0.3, traces of $ZnSO_4$;$MnSO_4$;$FeSO_4.7H_2O$ and carbon source (CMC or milled lignocellulosic biomass) 10.0. The pH of the basal medium was adjusted to 6.8. The media were than autoclaved for 20 minutes at 121°C.*B. subtilis* cultivated in sterile enzyme production medium containing Carboxy methylcellulose (CMC). Fermentation was carried out in 250 ml Erlenmeyer flasks, each containing 100 ml sterile production medium and inoculated with 2% of standard inoculums (containing 2-3.5x106 cells/ml). The flasks were incubated at 37°C for bacteria on a rotary shaker at 150 RPM for 72h. (Shaikh*et al*., 2013)

Enzyme Preparation and Enzyme assay

Samples (5ml) of culture broth were taken aseptically at regular intervals throughout the growth phase, centrifuged at 5000 Xg for 8 minutes and the supernatant used as the crude extra cellular enzyme extract. The assay of cellulose activity was performed by measuring the release of reducing Sugar (RS) by Dinitrosalicylic acid (DNSA) method of Miller *et al.* (1960) with glucose as a standard.

β-1, 4 – Glucanglucanodydrolase (Endoglucanase or CM Cellulaseor EG) :

The enzyme activity was measured by adding 1.4 ml. of 0.5 M Citrate-phosphate buffer (pH4.8) and 0.1 ml. of appropriately diluted enzyme to 0.5 ml. of 1% aqueous solution of carboxymethyl cellulose (CMC). The reaction mixture was incubated at 50^0C for 20 minute and the amount of reducing sugar produced was determined. Absorbance was read at 540nm against broth as reference. For endoglucanase the amount of reducing sugar was estimated from a glucose standard curve. One unit of enzyme activity was expressed as the

amount of enzyme which produced 1µ mole of reducing sugar per minute (1 IU = 1µ mole / minute of glucose equivalent released or 0.18 mg/minute of glucose

Effect of Incubation Period on Enzyme Production

Tooptimizedthe fermentation period media was incubated with *Bacillus subtilis* for different time intervals of 12 hours up to 120 hours. Hydrolysis of cellulosic materials estimated in terms of reducing sugar and endoglucanase activity occurred in hydrolysing broth.

Effect of Temperature on Enzyme Production

In order to determine the effective temperature for cellulase production by *Bacillus subtilis*fermentation was carried out at different temperatures (20°C,25°C, 30°C, 37°C, 45°C, 55°C) and Enzyme activity and production of reducing sugar were recorded.

Cellulose production at different pH

Cellulase enzyme producing broth was prepared and pH was adjusted at 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. *B. subtilis* grown in media Enzyme activity and production of reducing sugar were recorded.

Effect of inoculum concentration

Bacillus subtilis, cultivated in standardised enzyme producing medium with different inoculum concentration i.e., 0.5%, 1%, 2%, 3%, 4% (v/v) and reducing sugar and cellulase production were observed.

Effect of different Carbon Source

Different carbon sources like Maltose, Dextrose, Glucose, Fructose, Lactose, Sucrose and Xylose were analysed for the cellulase production. Various concentrations of carbon sources were used to replace 1% carbon source which was the original concentration in growth media with 1 to 5%. Carboxymethyl cellulose (CMC) also used as control.

Effect of different Nitrogen Source

The effect of different Nitrogen sources on the production of cellulase enzyme by *Bacillussubtilis*was investigated. The nitrogen sources tested ranged from 0.5 to 4.0% (w/v) Yeast extract, Peptone, Urea, Casein, Tryptone, Ammonium sulphate, Ammonium chloride, Potasium and Sodium nitrate.

Effect of Agro-Based Waste Material

*Bacillus subtilis*successfully produced cellulases and were used for saccharification of agro-waste materials. The strain incubated with 9 different types of agro waste materials (Sugarcane bagasse, Banana stalk, Corn cob, Coconut coir, *Eichhornia*, Groundnut shell, *Parthenium* stalk, Rice straw and Wheat straw along with CMC as a control).

Consortia applied on different lignocellulosic materials

Mix culture of *Bacillus subtilis, Cellulomonas sp.* and *Pseudomonas sp.* grown on 1% lignocellulosic materials, Rice Straw, Bagasse, Corncob, Coconut coir and Wheat straw.

Statistical analysis

Protein contents, enzyme activities and reducing sugar estimation were always carried out with a minimum of three replicates. Standard deviation was based on spreadsheet calculation using the Excel suite available in the Microsoft® Office 2010 software package (Microsoft, Redmonde, WA). Error bars represent the standard deviation of each experimental data point.

RESULTS AND DISCUSSION

Effect of incubation time

Maximum endoglunase activity observed at 48 hours (0.56±0.05 IU/ml) in the broth of *B. subtilis* activity was almost stable up to 60 hours (**Fig 1**). Enzymatic activity decreased gradually after optimum time simultaneously amount of reducing sugar appear after just 12 hours of incubation and increased gradually up to observation time. The cellulase activity was significantly reduced after 96 hours due to depletion of nutrients or accumulation of other by products in the fermentation media which lead to decrease in cellulase activity (Das *et al*., 2010). Production of enzyme is concerned with production of primary metabolites and it was directly related with growth phases of organisms. Organism showed their maximum activity according to their different time of log phase. The maximum cellulase production of *Thermophilicbacillus* sp. after 96 hours of incubation was also obtained by Haq*et al*. (2005).

Effect of Incubation Temperature on enzyme production

Temperature plays a crucial role in growth and physiology of microorganisms and its enzyme activity. Enzyme activity and production of reducing sugar recorded at different temperatures (20°C,25°C, 30°C, 37°C, 45°C, 55°C) revealed that *Bacillus subtilis* produced maximum cellulase and reducing sugar at 37±2°C (**Fig 2**). The reduction of enzyme activity was obtained with further increase in temperature. The increase in temperature, above the optimum values, results in loss of enzyme activity due to thermal denaturation of enzymes, hence low enzyme activity was observed above 45°C.Similar results of maximum cellulase production of 0.5851 ± 0.006 IU/mL was achieved after 72 hours of incubation at 37°C from *Bacillus pumilus*EWBCM1 (Shankar and Isaiarasu 2011). Sreedevi et al. (2013) also reported 37°C as an optimum temperature for cellulase production by *Bacillus* sp. BSS3.

Effect of pH on enzyme production

The production media with different pH values (4.0, 5.0, 6.0, 7.0, 8.0) were used for growth of *Bacillus subtilis*to investigate its effect on reducing sugar and cellulase production. pH 7.0 was found as optimum pH for production of reducing sugar and cellulase enzyme (**Fig. 3**).The result was supported by finding of Lokhande and Pethe (2017). For the production of cellulase by *Bacillus subtilis* and *Bacillus circulans*, the pH in the range of 7.0-7.5 was found to be optimum by Ray *et al*. (2007). The optimum pH for the maximum cellulase production was found to be 7.0 for cellulase production by *Bacillus* sp. 8 and *Bacillus* sp. 17 by Nasr *et al*. (2011) also. The maximum CMCase activity was reported in a study on cellulase production by *Bacillus* sp. at pH 7 (Padilha*et al*., 2015).

Effect of inoculum concentrationon enzyme production

Bacillus subtilis, cultivated in standardised enzyme producing medium with different inoculum concentration i.e., 0.5%, 1%, 2%, 3%, 4% (v/v) and reducing sugar and cellulase production were observed. Nutrient broth grown and 24 hour old bacterial strains were used as Inoculum. The optimum inoculum concentration for cellulase production was recorded at 2% (v/v) in terms of maximum reducing sugar and endoglucanase activity (**Fig.4**).

After the optimal inoculum concentration, the enzyme activity was sharply reduced because microbial growth was decreased due to increase in competition for space and nutrients among cells. These factors also affect the length of stationary phase, which results in loss of enzyme activity due to accumulation of toxic products and secondary metabolites.Similar results of maximum cellulase production were also reported by Acharya and Chaudhary (2011). In another study given byShankar and Isaiarasu(2011) on cellulase production by *Bacillus pumilus*,2% (v/v) inoculum size was found to be optimum for maximumcellulase production.

Effect of different Carbon Sourceon enzyme production

Nutrient sources were found to be the next important factor for the Cellulase production. Since carbon is considered as the primary nutrient for the bacteria, different carbon sources like Maltose, Dextrose, Glucose, Fructose, Lactose, Sucrose and Xylose were analysed for the cellulase production. Various concentrations of carbon sources were used to replace 1% sugar which was the original concentration in growth media with 1 to 5%. Carboxymethyl cellulose (CMC) also used as control. Results obtained showed that 5% carbon source brought the highest production of reducing sugar and cellulase enzyme at 48 hours of incubation (**Table – 1**). Enzymatic activity decreased in the presence of glucose, fructose and xylose and increased in the presence of Maltose, dextrose, lactose and sucrose as compare to control but result was not very significantly difference. Reducing sugar also produced accordingly. Catabolite repression plays an important role in the regulation and secretion of inducible enzyme. Such repression effect has been observed in other organisms (Magnelli and Forchiassin, 1999; Hrmova*et al*., 1991). When soluble non reducing sugars such as glucose fructose and xylose were utilized as sole carbon sources, significantly cellulase activity could not be detected in fermented broth. However, some reducing sugars would not be induced to produce cellulase after their reducing groups were substituted by hydroxyl groups to become sugar alcohol like mannitol, sorbitol, and xylitol (Hong *et al*., 2013). Teodoro*et al*. (2000) also reported Maltose as the best carbon source for *Bacillus* sp.

Effect of different Nitrogen Sourceon enzyme production

Nitrogen is one of the important elements required for growth of microorganisms. Provision of utilizable form of nitrogen source to organisms is the basic requirement to be fulfilled for optimal growth. In order to find out the best utilizable form of nitrogen source for growth, extracellular protein and cellulase production by bacterial strains on enzyme production medium supplemented with different nitrogen sources Yeast extract, Peptone, Urea, Casein, Tryptone, Ammonium sulphate, Ammonium chloride, Potassium and Sodium nitrate [tested ranged from 0.5 to 4.0% (w/v)]. The results in **Table - 2** showed that a concentration of 2.0% organic Nitrogen source (Yeast extract, Peptone, Urea, Casein, Tryptone) and 3% inorganic nitrogen source (Ammonium sulphate, Ammonium chloride, Potassium and Sodium nitrate) were optimum for production of reducing sugar and cellulase enzyme. Peptone (0.59±0.05 IU/ml) was the best nitrogen source for cellulase production for *Bacillussubtilis*followed byTryptone.

The presence of external nitrogen source is essential in the fermentation media during extracellular enzyme production for effective utilization of soluble carbohydrates. The use of organic nitrogen sources as compared to inorganic sources for maximum cellulase production was found to be more suitable for maximum cellulase production (Ariffin*et al*., 2008). Similarly the highest cellulase production was found with *Bacillus subtilis* by utilizing peptone as nitrogen source.

Effect of Agro-Based Waste Materialon enzyme production

Bacillus subtilis, Cellulomonas sp. and Pseudomonas sp. successfully produced cellulases and were used for saccharification of agro-waste. The cellulolytic enzyme producer bacterial strains incubated with 9 different types of agro waste materials (Sugarcane bagasse, Banana stalk, Corn cob, Coconut coir, *Eichhornia*, Groundnut shell, *Parthenium* stalk, Rice straw and Wheat straw along with CMC as a control). The degree of saccharification was assayed on the basis of release the amount of reducing sugar and cellulase activity. The observation performed after 36 hours which was optimum time for cellulase production. Maximum to least production of reducing sugar and cellulase enzyme followed the following order by *B. subtilis*. Result tabulated in **Table –3**.

Rice straw>Sugarcane bagasse>Corn cob > Banana stalk >Parthenium stalk >Eichhornia> Groundnut shell > Coconut coir > Wheat straw

Production of reducing sugar and cellulase enzyme reduced greatly in the presence of wheat straw, coconut coir and ground nut shell as compare to control. Rice straw, Sugarcane bagasse, Corn cob, Banana stalk, *Parthenium*stalk and*Eichhornia* were good stimulator of production of reducing sugar as well cellulase enzyme. The biological process for converting lignocellulose into fermentable sugars requires delignification to liberate cellulose and hemicelluloses from their complex with lignin and depolymerization of the carbohydrate polymers to produce free sugars in the form of pentoses and hexoses (Bansal*et al*., 2012).

Consortia applied on different lignocellulosic materials

Mix culture of *Bacillus subtilis, Cellulomonas sp.* and *Pseudomonas sp.* grown on 1% lignocellulosic materials, Rice Straw, Bagasse, Corncob, Coconut coir and Wheat straw. Maximum hydrolysis observed in rice straw and minimum digestion occurred in coconut coir. Baggase and corn cob were also good substrate for endoglucanase and reducing sugar (**Fig. 5**). The advantages of employing mixed cultures as opposed to pure cultures in bioremediation have been widely demonstrated. It could be attributed to the effects of synergistic interactions among members of the association. It is possible that one species removes the toxic metabolites (that otherwise may hinder microbial activities) of the species preceding it. It is also possible that the second species are able to degrade compounds that are partially degraded by the first (Kumar *et al.,* 2008). Various reports are available on the study of microbial consortia for their biotechnological application. Sarunyou*et al.,* (2010), showed degradation of lignocellulosic agro-industrial residues by means of complex microbial community and had shown it as a promising approach providing efficient biomass decomposition for subsequent conversion to value added products.

CONCLUSION

The results of present study described, *Bacillus subtilis*exhibited great potential for production of cellulase enzyme. The optimised nutritional and environmental parameters increased capacity to produced enzyme. The organisms also proved to be an excellent source for saccharification of lignocellulosic waste to ethanol production and contribute to solve the problem of fuel crisis. Apart from ethanol production *B. subtilis* is used for of Municipal Solid Waste, Pharmaceutical industries and Agricultural industries.

REFERENCES

Acharya S, Chaudhary A (2011) Effect of nutritional and environmental factors on cellulases activity by thermophilic bacteria isolated from hot spring. *J. Sci. Ind. Res*., 70: 142-148.

Ariffin H, Hassan MA, Shah UK, Abdullah N, Ghazali FM, et al. (2008) Production of bacterial endoglucanase from pretreated oil palm empty fruit bunch by *Bacillus pumilus* EB3. *J. Biosci. Bioeng*., 106:231-236.

Bansal N, Soni R, Janveja C et al. (2012) Xylanase-cellulase complex from *Bacillus.Lignocellulose*., 1(3),:196-209.

Bergey's Manual of Systematic Bacteriology Volume One : The Archaea and the Deeply Branching and Phototrophic Bacteria Editors: Editor-in-chief: Garrity, George Boone, David R., Castenholz, Richard W. (Eds.)(2001 edition).

Bhat MK (2000) Cellulases and related enzymes in biotechnology.*Biotechnol. Adv*., 18(5): 355–383.

Cappuccino JC, Sherman N (1992) Microbiology: A Laboratory Manual (third ed), Benjamin/ cummings Pub. Co., New York. pp. 125-179.

Das A, Bhattacharya S, Murali L (2010) Production of cellulase from thermophilic*Bacillus* sp. isolated from cow dung. *AM Eurasian J. Agric. Environ. Sci*., 8: 685-691.

Haq IU, Hameed U, ShahzadiK,Javed M, Ali S, et al. (2005) Cotton saccharifying activity of cellulases by *Trichodermaharzianum* UM-11 in shake flask. *Int. J.Bot*. 1: 19-22.

Hong Y, Yingjie D, Xin B, Cailian Y, Wenting W, Dexin S, Liqiong Y, Haining W, Dan L, Yujie F (2013) Characterization of cellulase production by carbon sources in two *Bacillus* species. *Afr. J.Biotechnol*. 12(48): 6745-6753.

Hrmova M, Petrakova E, Biely P (1991) Induction of cellulose- and xylan-degrading enzyme systems in *Aspergillusterreus* by homoand hetero-disaccharides composed of glucose and xylose. *J. Gen.Microbiol*., 137: 541-547.

Immanuel G, Dhanusha R, Prema P, Palavesam A (2006) Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. *Int. J. Environ. Sci. Technol*., 3(1):25-34.

Jarvis M (2003) Cellulose stacks up. *Nat*., 426 (6967): 611–612.

Kumar M, Leon V, Materano ADS, Ilzins OA, Luis L (2008) Biosurfactant production and hydrocarbon-degradation by halotolerant and thermotolerant Pseudomonas sp. *World J. Microbiol. Biotechnol.*, 24: 1047-1057.

Lee JW, Kim HY, Koo BW, Choi DH, Kwon M, Choi IG, Lee JW, Kim HY, Koo BW, Choi DH, Kwon M, Choi IG (2008) Enzymatic saccharification of biologically pretreatedPinusdensiflora using enzymes from brown rot fungi. *J. Biosci. Bioeng*., 106(2): 162-167.

Lokhande S,Pethe AS (2017) Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production. *Int. J. of Life Sciences.*, 5 (2): 277-282

Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: Fundamentals and biotechnology.*Microbiol.Molecul. Biol. Rev*., 66(3): 506-577

Magnelli P, Forchiassin F (1999) Regulation of the cellulase complex production by *Saccobolussaccoboloides*: induction and repression by carbohydrates. *Mycologia*., 91 (2): 359–364.

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar.*Analytical Chemistry*, 31(3) : 426 – 428.

Nakamura K, Kppamura K (1982) Isolation and identification of crystalline cellulose hydrolysing bacterium and its enzymatic properties. *J. Ferment. Technol*., 60 (4): 343–348.

Nasr SA, Abozaid AA, Hussein NA, Al-Salemi FA (2011) Cellulase production by local bacteria isolated from Taif in Saudi Arabia. *J. Agri. Sci*., 19: 163-170.

Padilha QM, Carvalho LCT, Dias PVS, Grisi TCSL, da Silva FLH, et al. (2015) Production and characterization of thermophiliccarboxymethyl cellulase synthesized by *Bacillus* sp. growing on sugarcane bagasse in submerged fermentation. *Braz. J. Chem. Eng*., 32: 35-42.

Ray AK, Bairagi A, GhoshKS,Sen SK (2007) Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut. *Actalchthyologica ET Piscatoria*., 37: 47-53.

Sarunyou W, Ukrit R, Thanaporn L, Lily E, Yasuo I, Verawat C (2010) Analysis of a thermophilic lignocellulose degrading microbial consortium and multi-species lignocellulolytic enzyme system. *Enz.Microbiol.Technol*., 47.283-290.

Shaikh NM, Patel AA, Mehta SA, Patel ND (2013) Isolation and Screening of Cellulolytic Bacteria Inhabiting Different Environment and Optimization of Cellulase Production. *Uni. J. Environ. Res. Technol*., 3(1) : 39 – 49.

Shankar T, Isaiarasu L (2011) Cellulase production by *Bacillus pumilus* EWBCM1 under varying cultural conditions. *J. Sci. Res*., 8: 40-45.

Shin CS, Lee JP, Lee JS, Park SC (2000) Enzyme production of *Trichodermareesei* rut C-30 on various lignocellulosic substrates. *Appl. Biochem. Biotechnol*., 84(86): 237–245.

Sreedevi S, Sajith S, Benjamin S (2013) Cellulase producing bacteria from the wood-yards on kallai river bank. *Adv. Microbiol*., 3: 326-332.

Teodoro S, Martins L(2000) Culture conditions for the production of thermostable amylase by *Bacillus* sp. *Braz. J.Microbiol*., 31: 298-302

Tomme P, Warren RAJ, Gilkes NR (1995) Cellulose hydrolysis by bacteria and fungi.*Advance.Micro. Phys*., 37: 1–81.

Xu W, Shi L, Chan O, Li J, Casper P, Zou X(2013) Assessing the effect of litter species onthe dynamic of bacterial and fungal communities during leaf decomposition inmicrocosm by molecular techniques. *PLoS One*., 8:e84613.

Zhang Y-HP, Lynd LR (2004) Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulose systems. *Biotechnol.Bioeng*., 88: 797-824.