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Production of Cellulase – A Review

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Abstract: ***Production of celluases at commercial level is most actively grown area of research now a day. Screening of potential strain from new source and there by optimizing production condition for industrial cellulase. Cellulase is one of the several commercial enzymes which have been used in various industries like paper and pulp, texile, bio-fuel production, detergents, feed and food industry and brewing etc. The review discusses the current knowledge on cellulase production by bacteria. It discuses the industrial application of cellulases and challenges in cellulase research especially in direction of improving the process economics enzyme production.***

Keywords: cellulose, detergent, brewing, biomass, biofule, cellulose, municipal waste

1. Introduction

Cellulase is the most abundant biomass; it is renewable and in-expensive for the bioconversion to bio-fuels and bio-products. There are many sources to derive cellulosic biomass from such municipal waste, agricultural residues, forestry or pulp and energy crops. Cellulose is commonly degraded by enzyme called cellulase. Cellulase refer class of enzymes produced chiefly by fungi, bacteria and protozoans that catalysis the cellulose. Due to its diversity of their application cellulase have attracted much interest. The major industrial application of cellulases are in textile industry for bio-polishing of fabrics, house-hold laundry detergents for improving fabrics softness and brighteness. Cellulases hydrolyze cellulose and produces primary products glucose, cellbiose and cello-oligosaccharides. There are three types of cellulase enzymes [cellbiohydrolase(CBH), endo-β1,4-glucanase(EG) and β- glucosidase]. Enzymes within these classification can be separated into individual components, such as microbial cellulase composition may consist of one or more CBH component, one or more EG components and possibly β- glycosidase. Commercial production of cellulase has been tried by either solid or submerged culture including batch, fed batch and continuous flow process. Media used in cellulase fermentation contain cellulose in different degrees of purity, or as raw lignocellulosic subtrates, which is especially true in solid state fermentation**.**

Cellulases

Cellulase (EC3.2.1.4) refers to a class of hydrolases produced mainly by fungi ,bacteria, protozoans, and termites, which catalyzes the hydrolysis of cellulose (Lee *et al*., 2000; Watanabe *et al.*, 1998). However, there are also cellulases produced by other types of organisms such as plants, molluscs, animals (Watanabe and Tokuda, 2001). This type of cellulase is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores.

Recently, following the report of an endogenous cellulose gene in termites, which were previously considered to digest cellulose exclusively through symbiotic protists (Watanabe et a., 1998), endogenous genes have also been found in many invertebrates such as insects, nematodes and molluscs (Watanabe and Tukuda, 2001). These findings contradict previously held notions that cellulose can only be degraded by micro organisms.

Cellulose decomposition or degradation requires the multiple emzymes, celluloses. In general, cellulose is degraded to cellodextrins or glucose by the sequential synergistic action of three cellulose systems: end-1-4-β-glucanase, exo-1,4-β-glucanase, and β–glucosidase (Bayer et al., 1998; Henrissat, 1994).

Endo-1, 4-β-glucanase (EC 3.2.1.4)

Endo-1, 4-β-glucanase (EG), simply called endoglucanase, cleave randomly intermolecular β-1, 4-glucosidic linkages within thecellulose chain. The endoglucanases are commonly assayed by viscosity reductions in carboxymethyl cellulose (CMC) solution. Themodes of actions of endoglucanases and exoglucanases differ in that endoglucanases decrease the specific visocosity of CMC significantly with little hydrolysis due to intramolecular cleavages, whereas exoglucanases hydrolyze long chains from the ends in aprogressive process (Teeri, 1997; Zhang and Lynd, 2004).

Exo-1, 4-β-glucanase (EC 3.2.1.91)

Exo-1, 4-β-glucanases (exo-1, 4-β-D-glucan cellobiohydrolases, CBH), simply called exoglucanases, cleave the accessible ends of cellulose modules to liberate glucose and cellobiose. *Triochoderma reesei* cellobiohydrolase I and II act on the reducing and nonreducing cellulose chain ends respectively (Teeri, 1997; Teeri, *et al*., 1998). Avicel has been used for measuring exoglucanase activity among insoluble cellulosic substrates. Unfortunately, amorpHous cellulose and soluble collodextrins are substrates for both purified exoglucanases and endoglucanases. Therefore, unlike endoglucanases and β-glucosidases, there is no substrates specific for exoglucanases within the cellulase mixtures (Sharrock, 1988). However, the enzymatic deploymerization step performed by endoclucanases and exoglucanases is the rate-limiting step for the cellulose hydrolysis process.

1,4-β-Glucosidase (EC 3.2.1.21)

β-D-glucosidases hydrolyze soluble cellobiose and other cellodextrins with a degree of polymerization (DP) up to six to produce glucose in the aqueous pHase. The hydrolysis rate markedly decreases as the substrate degree of polymerizations increases (Henrissatet al., 1989; Zhang and Lynd, 2004). The term “cellobiase” is often misleading due to this key enzymes broad specificity beyond a DP of two. Relative to endoglucanaises and celobiohydroloses, low

levels of the *T. reesei* β-glucosidase are selected in submerged culture.

Substrates for Cellulase Activity Assays

Soluble:
Long chain cellulose derivatives: CMC, HEC.

Short chain (low DP): Cellodextrins, Radio-labelled cellodextrins .
Cellodextrin derivatives:β -methylumbelliferyl-oligosaccharides. p-nitropHenol oligosaccharides.

Insoluble:
Crystalline cellulose:cotton microcrystalline,Cellulose (Avicel), veloniaCellulose, bacterial cellulose.
AmorpHous cellulose:PASC, alkali-swollen cellulose,RAC.

Keys: RS = reducing sugars, CMC = Carboxymethyl Cellulose; HEC = Hydroxymethyl cellulose; RAC = Regenerated amorpHous cellulose.

Soluble Substrates
Soluble substrates include low DP cellodextrins from two to six sugar units and their derivatives, as well as long cellulose derivatives.

They are often used for measuring individual cellulase component activity.

CMC-Na(sodium carboxymethyl celluloose) customarily shorten form CMC ,is easy to absorb moisture,it dissolve easly in cold or hot water as colloidal solution, it can dissolve in many organic factor solvent such as methanol, ethanol,aceton chloroform and so on.digree of substitution(D.S) is an important factor that affects it water-soluble,viscosity also affects its water soluble greatly.CMC ia water soluble when its D.S more than 0.4.with increase of D.S transparency of the CMC water substitution becomes much better.

Chromogenic p-nitropHenyl glycosides and fluorogenic methylumbelliferyl-D-glycosides derived from soluble cellodextrins are very useful for the study of initial cellulase kinetics (Tuohy *et al*., 2002), reaction specificity (Zverlov et al., 2002), and binding sitethermodynamics (Barr and Holewinski, 2002). They are also used to determine the inhibition constants of cellulase in the presence of addedcollobiose and glucose (Tuohy *et al*., 2002), because chromopHores released form substitutedglycosides can be easily measuredindependently of sugars.

Insoluble Substrates
Insoluble cellulose-containing substrates for cellulase activity assays include nearly pure celluloses (Cotton linter, Whatman No.1 filter paper, bacterial cellulose, microcrystalline cellulose and amorpHous cellulose) and impure cellulose – containing substrates(dyed cellulose, α-cellulose, and pretreated lignocellulose). Native cellulose, referred to as cellulose I, has two distinct crystalline forms-Iα, which is dominant in bacterial and algal cellulose, Iβ which is dominant in higher plants (Atalla and Vanderhart, 1984).

Native cellulose (cellulose I) can be converted to other crystalline forms (II-IV) by various treatments (O'Sullivan, 1997). Several very pHysical values such as crystalinity index (CrI), degree of polymerization, and cellulose accessibility to cellulose, can be estimated based on maximum cellulase adsorption (Zhang and Lynd, 2004).

Lignocellulose pretreatment breaks up the recalcitrant structure of lignocellulose so that cellulase can hydrolyze pretreated lignocelluose faster and more efficiently. Current leading lignocelulose pretreatment technologies, including dilute acid, hot water,flow through ammonia fiber explosion (AFEX), ammonia recycle percolation, and lime, have been recently reviewed (Mosier *et al*.,2005; Wyman *et al*., 2005).Other insoluble substrates include α–cellulose which contains major cellulose, and a small amount of hemicelluloses and dyed cellulose. Insoluble cellulose derivatives can be chemically substituted with trinitropHenyl groups to produce chromogenic trinitropHenyl-carboxymethyl cellulose (TNP-CMC).

Approaches for cellulase activity assay

Two approaches to measure cellulose activity are:
- Measuring the individual cellulase (endoglucanse, exoglucanase, and beta glycosidase) activites
- Measuring the total cellulase activity

Endoglucanase activity assay can be measured on reduction in substrate viscosity or increase in reducing end determined by reuding sugar assay.

In Exonuclease activity assay Avicel has been used for measuring exoglucanase activity. During chromatograpHic fractionation of cellulase mixtures, enzymes with little activity on soluble CMC but showing relatively high activity on avicel, are usually identified as exoglucanases. Unfortunately, amorpHous cellulose and soluble cellodextrins are substrates for both purified exoglucanases and endoglucanases. Therefore, unlikeendoglycanases and β-glucosidases, there is no substrates specific for exoglucanases within the cellulase mixture (Sharrock, 1998; Wood and Bhat, 1988).

β-D-glucosidase are very amenable to a wide range of simple sensitivity assay methods, based on coloured or fluorescent products from p-nitropHenyl- β-D-1,4-glucopyranoside (Strobel and Russell, 1987), β-napHythyl-β-D-glucopyranoside, 6-bromo-2-napHthyl- β-D-glucopyranoside (Setlow *et al*., 2004). Also, β-D-glucosidase activities can be measured using cellobiose, which is not hydrolyzed by endoglucanases and exoglucanases, and using longer cellodextrins, which are hydrolyzed by endoglucanases and exoglucanases (Ghose, 1987; McCarthyl *et al*., 2004).

The total cellulase system includes endoglucanases, exoglucanases, and β-D-glucosidases, all hydrolyze cellulose synergistically. Total cellulase activity assays are always measured using insoluble substrates, including pure cellulosic substrates such as Whatman No.1 filter paper, cotton linter, microcrystalline cellulose, bacterial cellulose, algal cellulose and cellulose containing substrates such as dyed cellulose, α-cellulose and pretreated lignocellulose.

Screening and isolation of cellulase producing bacteria:
Cellulase producing bacteria have been isolated from wide variety of sources such as composting heaps, decaying plant material from forestry or agriculture waste, faces of ruminants such as baffalow, caow, goat, and oraganic compund etc. screening for celluase production can be done by enrichment of growth on microcrystalline cellulose. screening for bacterial cellulase activity in microbial isolates is typically performed on CMC media followed by using congo red.

The isolation and identification of cellulase has been limited in past to culturable microorganisms. However recent advances in molecular techniques ,such as creation of metagenomic libraries will widen the pool of cellulolytic enzymes available for biofuel research.

Novel cellulase producing bacteria:
Isolation, screening and selection have favored the discovery of several novel cellulase producing bacteria from wide variety of environments. Due to vast diversity among bacteria the identification of novel cellulases remains a currently explored route to the improvement of biorefining industries. Recently, the bacterial strain B39, previously isolated from poultry manure compost in Taichung Taiwan, was identified through 16S Rrna gene sequencing and pHylogentic analysis to be a novel cellulose degrading *Paenibacillus sp*. Strain.

More recently, a thermostable cellulase was found in newly isolated *Bacillus subtilis* DR ,extracted from hot spring.

Table 1: Cellulase producing bacterial strain

Bacterial strain	Reference
Ruminococcus albus	Varel,1984,ohmiya et al, 1985
CytopHaga hutchinsonii	Clifford loumiya, 1980
succinogens	Groleu et al.,1981
Acetivibrrio cellulolytieus	Saddler et al., 2006
Branchamella	Ekperigin,MM.,et al., 2006
Acinetobacter anitratus	Ekperigin,mm., etal., 2006
Pseudomonas sp.	Yamane et al 1970., yoshikawa et al., 1974
Geobacillus pallidus	Azhari samsu baharuddin et al., 2010
Bacillus subtillis ko	Mohammed S.A. shabab et al,. 2010
Bacillus licheniformis mvs	Somen acharya et al.,2012

Improvement of bacterial celluases:
Despite the broad spectrum of cellulases being isolated, no single enzyme is completely suitable it is, for the hydrolysis of cellulose in biorefining industry. However these enzyme offer a good starting point for the improvement of cellulase in step toward enhancing overall economics of biofuel production. The use of protein engineering technology has been directed toward study of cellulase catalytic function. Modification of bacterial cellulase through the use of protein engineering is taking a stage in production of efficient hydrolytic enzyme used in broad scope of industries.

Table 2: Table of bacterial strain and cellulases or related enzyme from these microorganism which have been improved

Bacterial strain	*Enzyme*	*Property alterd*	*Method*	*Reference*
Acetothermus cellulolytics	Endoglucanase	Type of products released	Site directed mutagensis	Rignall TR, Baker Appl biochem botechnol . 2005
Acidothermus cellulolytics	Endoglucanase	Product inhibiton	Site directed mutagensis	Baker jo, Mc Carley JR.Appl biochem botechnol 2005
Pectobacterium chrysanthami	Endoglucanase	Activity	Nonsense mutation	Lim WJ, Hong SY,An jam. Appl biochem botechnol 2005
Thermobifida fusca	Processice endoglucanase	Activity	Site directed mutation	Escover –Kousen JM,Wilson D. 2004
Thermotoga maritima	Endoglucanase	Activity	Site directed mutation	Mhadevan SA, wi SG,Lee DS.2008
Bacillus subtilis	Endoglucanse	Activity	DNA sufflig	Kim YS, Jung HC.2000
Agrobacterium sp.	Mutated α-glucosidase	Activity	epPCR	Kim YS. Lee SS. 2004

Cellulase Production Using The Submerged Fermentation (SmF) and Solid State Fermentation (SSF) or Cultivation (SSC):

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms. It has been widely used for the production of cellulase for their wide uses in industry. Over the years, fermentation techniques have gained immense importance due to their economic and environmental advantages. Two broad fermentation techniques have emerged as a result of this rapid development:

Submerged Fermentation (SmF) and Solid State Fermentation (SSF).

Solid-State Fermentation (SSF) / Solid-State Cultivation (SSC)
SSF utilizes solid substrates, like bran, bagasse, paddy straw, other agricultural waste and paper pulp [**Subramaniyam R, Vimala R. Solid state and submerged fermentation for the production of bioactive substances: A comparative study. Int J Sci Nature.2012;3(3):480-486.**]. The main advantage of using these substrates is that nutrient-rich waste materials can be easily recycled as cheaper substrates. SSF is best suited for fermentation techniques involving fungi and microorganisms that require less moisture content. However, it cannot be used in fermentation processes involving organisms that require high water activity, such as bacteria [**Babu KR, Satyanarayana T. Production of bacterial enzymes by solid state fermentation. J Sci Ind Res. 1996;55:464-467.].**

Submerged Fermentation (SmF)/Liquid Fermentation (LF):

SmF utilizes free flowing liquid substrates, such as molasses and broth **[Subramaniyam R, Vimala R. Solid state and submerged fermentation for the production of bioactive substances: A comparative study. Int J Sci Nature.2012;3(3):480-486.].** This fermentation technique is best suited for microorganisms such as bacteria that require high moisture content. An additional advantage of this technique is that purification of products is easier.

A Comparison between SmF and SSC Method

Cellulases are produced using the submerged fermentation (SmF) method traditionally, in which the cultivation of microorganisms occurs in an aqueous solution containing nutrients. An alternative to this traditional SmF method is the solid state cultivation (SSC) method, which involves the growth of microorganisms on solid materials in the absence of free liquids **[Cannel E, Young MM. "Solid-State cultivation systems." Process Biochemistry. 1980; June/July: 2-7.]**. Since SSC involves relatively little liquid when compared with SmF, downstream processing from SSC is theoretically simpler and less expensive . During the past ten years, a renewed interest in SSC has developed due, in part, to the recognition that many microorganisms, including genetically modified organisms (GMO), may produce their products more effectively by SSC **[Pandey A, Selvakumar P, Soccol CR, Nigam P. Solid state cultivation for the production of industrial enzymes. Current Science. 1999;77:149-162.].**

Table 3: Comparison of Characteristics of SmF and SSC Mehtods

Factor	SmF	SSC
Water	High volume of water consumed and effluents discarded	Limited consumption of water and no effulent
Mechanical agtation scale up	Good homogenization industrial equipment available	Static condition preffered New design equipment needed
Energy	High energy consuming	Low energy comsuming

Table 4: Fermentative production of cellulase by bacteria

Name of bacteria	*temperature*	*Type of substrate used*	*pH*	*Type*	*Refernce*
Anoxybacillus flavithermus EHP2	75°C	CMC	7.5	SmF	Ibrahim ASS, Ahmed IED. Australian Journal of Basic and Applied Sciences. 2007;1(4):473-478.
Bacillus sp.AC-1	70°C	CMC	4.5–6.5	SmF	Li YH, Ding M, Wang J, Xu GJ, Zhao F..AC-1. Appl Microbiol Biotechnol. 2006;70:430-436.
Bacillus sp. LFC15	50°C		9–10	SmF	Korpole S, Sharma R, Verma D. Indian J Microbiol. 2011;51(4):531-535.
Bacillus sp		Round nut shell		SSF	Dey R, Pal KK, . Indian J. Microbiol. 2002;42:165-167.
Bacillus sp	50°C	Sugar Cane	4.5-5.5	SSF	Patel MA, Ou MS, Ingram LO, Shanmugam K. Biotechnol Prog. 2005;21:1453-1460
Bacillus sp. NZ	50°C	agricultural residues	9–10	SSF	Nizamudeen S, Bajaj BK. Food Technol. Biotechnol. 2009;47(4):435-440
Bacillus Cereus		Palm Kernel Cake		SSF	40. Lah NT, Rahman NB, Nama MB. International Conference on Environment, Energy and Biotechnology IPCBEE vol.33 (2012) © (2012) IACSIT Press, Singapore 172-177.

2. Conclusion

Cellulases were produced by SmF and SSF using various bacterial strains. Development of an economical process for cellulase production is hindered because of the high costs of substrate (pure cellulose) and of some chemicals, such as proteose peptone, and also because of low yields of cellulases per unit of cellulose. To overcome these bottlenecks, cheap source of cellulose; lignocelluloses, agricultural wastes are used in SSF.The microorganisms which appear to be most promising at present are *psudomonas sp*. However, it is of interest to examine *psudomonas sp*. to improve cellulaseproduction which is a known good producer of cellulases .Many researches have been conducted on enzymatic hydrolysis of various lignocellulolytic substrates like Pumpkin oil cake, Saw dust, Pine apple waste, Orange waste, Palm oil mill effluent, pea shrub biomass, Sugarcane bagasse, Rice bran, Rice straw, wheat bran, vinegar waste, Cassava waste, Corn straw, wheat straw, rice husk, soybean, , corn cob, green grass, dried grass, Millet, Oats straw, Oil palm biomass, Banana stalk, mulch, Radicle waste.

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