Improvement of biocatalytic efficacy of endoglucanase from indigenous strain, *Bacillus subtilis* MU S1 and its application in saccharification of agro-wastes

Thesis submitted to the
University of Calicut in partial fulfilment of the
requirements for the award of the degree of

DOCTOR OF PHILOSOPHY IN MICROBIOLOGY
Under the faculty of Science

By

C. P. SREENA

Under the guidance of

Dr. Denoj Sebastian

DEPARTMENT OF LIFE SCIENCES
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CERTIFICATE

This is to certify that the thesis entitled **"Improvement of biocatalytic efficacy of endoglucanase from indigenous strain, *Bacillus subtilis* MU S1 and its application in saccharification of agro-wastes"** is a bonafide research work done by Mrs. C.P. Sreena, under my supervision and guidance for the award of the degree of Doctor of Philosophy in Microbiology under the Faculty of Science of University of Calicut. I also certify that no part of the thesis has been presented before, for any other degree, diploma or associateship in any other University.

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DECLARATION

I, C.P.Sreena, hereby declare that the thesis entitled **"Improvement of biocatalytic efficacy of endoglucanase from indigenous strain, *Bacillus subtilis* MU S1 and its application in saccharification of agro-wastes"** submitted to the University of Calicut, for the award of degree of Doctor of Philosophy in Microbiology is a bonafide record of original work carried out by me under the supervision and guidance of Dr. Denoj Sebastian, Assistant Professor of Microbiology, Department of Life Sciences, University of Calicut and that it has not been submitted for the award of any other degree or diploma**.**

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C.P. Sreena

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LIST OF ABBREVIATIONS

AA	Auxiliary activities
AH	Arecanut husk
ANOVA	Analysis of variance
BCA	2,2′-bicinchroninate
BGL	β-glucosidase
BLAST	Basic Local Alignment Search Tool
BP	Banana peduncle
BSA	Bovine serum albumin
CAGR	Compound annual growth rate
CAZy	Carbohydrate-Active Enzymes
CBHs	Cellobiohydrolases
CBM	Carbohydrate binding module
CCD	Central composite design
CD	Catalytic domain
CE	Carbohydrate esterase
CIAP	Calf intestinal alkaline phosphatase
CMC	Carboxymethyl cellulose
CMCase	Carboxymethylcellulase
CT	Cocktail
DNS	Dinitrosalicyclic acid
DOE	Design of Experiments
EG	Endoglucanase
EtBr	Ethidium bromide
FPase	Filter paperase
GH	Glycoside hydrolase
Gor	Glutathione reductase
GST	Glutathione *S*-transferase
GT	Glycosyl transferase
IPTG	Isopropyl-β-D-thiogalactopyranoside
JOR	Jackfruit outer rind
MBP	Maltose-binding protein

MCS	Multiple cloning sites
MEGA	Molecular Evolutionary Genetics Analysis
mM	millimolar
NCBI	National Center for Biotechnology Information
NS	Nelson-Somogyi
NTA	Nitrilotriacetic acid
NusA	N-utilization substance protein
OFAT	One-factor-at-a-time
ORF	Open reading frame
OVAT	One-variable-at-a-time
PAHBAH	4-hydroxybenzoylhydrazine
PBD	Plackett-Burman design
PL	Polysaccharide lyase
PMSF	Phenylmethanesulfonyl fluoride
PW	Pepper waste
rpm	Revolutions per minute
RSM	Response surface methodology
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SmF	Submerged fermentation
SSF	Solid-state fermentation
SUMO	Small ubiquitin modifier
TBE buffer	Tris Borate EDTA buffer
TEV	Tobacco etch virus
TH	Tamarind husk
Trx	Thioredoxin
TrxB	Thioredoxin reductase

SREENA C. P. “IMPROVEMENT OF BIOCATALYTIC EFFICACY OF ENDOGLUCANASE FROM INDIGENOUS STRAIN, BACILLUS SUBTILIS MU S1 AND ITS APPLICATION IN SACCHARIFICATION OF AGRO-WASTES”. THESIS. DEPARTMENT OF LIFE SCIENCES, UNIVERSITY OF CALICUT, 2018.

Enzymes are biocatalysts that drive majority of the biochemical activities occurring in a cell. They are complex protein molecules produced by living organisms to facilitate specific chemical reactions essential for the life of the organism. In the absence of enzymatic catalysis, the biochemical activities will be so slow that they would not occur under the conditions compatible with life. Enzymes accelerate the rate of a reaction by about million folds, such that reactions requiring years, occur in fraction of seconds. They accomplish this by reducing the activation energy required to initiate the reaction. Enzymes are highly specific in their action and act only on certain type of substrates, without being consumed in the reaction. They are considered as green catalyst as they are non-toxic, biodegradable and can be produced in large amounts without the assistance of special chemical resistant- equipments (Smith 2009).

The term “enzyme” (literally ‘in yeast’) was coined by Wilhelm Friedrich Kühne in 1876. Enzymes are classified into six main categories: oxidoreductase, transferases, hydrolases, lyases, isomerases and ligases, based on the nature of chemical reaction they catalyze. Currently more than 3000 enzymes have been described. Although enzymes are formed in living organisms they continue to function in vitro with the same specificity, making them extremely useful in many industrial processes. The global market for industrial enzymes was estimated at $ 4.4 billion in 2015 and is expected to reach $ 6.30 billion by 2022 in terms of value, at a CAGR (compound annual growth rate) of 5.8 % from 2017 (Rohan

2017). Among the industrial enzymes approximately 75 % are hydrolytic. Protease represents the largest group of industrial enzymes followed by amylase and cellulase (de Souza et al. 2015). Cellulases are the third most important enzymes in the market due to its wide range of applications. However, the demand for cellulases is growing rapidly due to its role in production of glucose feedstock from the lignocellulosic materials, and this demand is the major incentive for cellulase research.

1.1. Cellulose: occurrence, structure and composition

Cellulose is most abundant and renewable biopolymer on earth. Every year about 10^{11} tons of dry plant material is generated worldwide as a consequence of photosynthetic fixation of CO^2, and almost 50 % of it is made of cellulose. In nature, cellulose is present in pure form in very few instances, as in cotton balls which has almost 100 % cellulose. But, in most cases cellulose fibers are embedded in a matrix of other structural biopolymers like hemicelluloses and lignin, which comprise 20 to 35 and 5 to 30 % of plant dry weight respectively (Lynd et al. 1999). Other than the three components, lignocellulosic materials also contain small amounts of pectin, proteins, extractives (i.e. non-structural sugars, nitrogenous material, chlorophyll and waxes) and ash (Kumar et al. 2009b) (Figure 1.1).

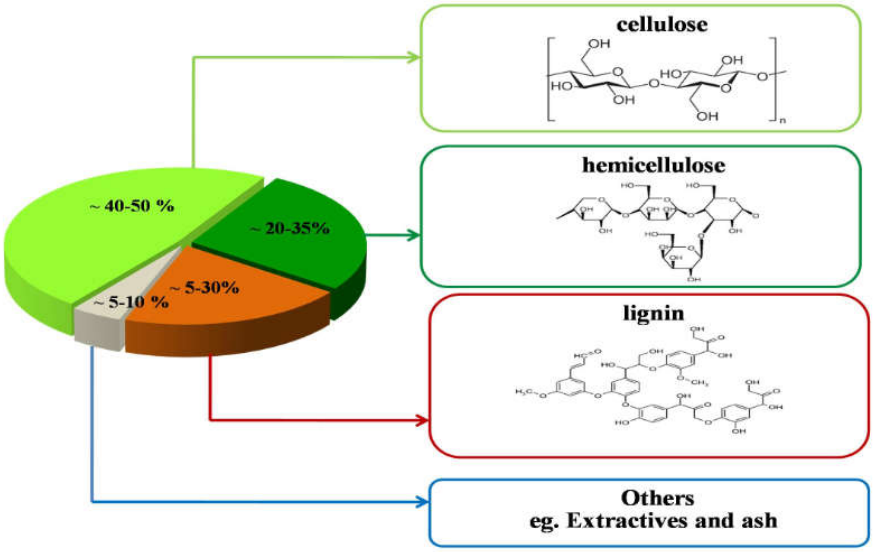


Figure 1.1: Composition of lignocellulosic biomass

French chemist Anselme Payen is credited with the discovery of cellulose. In the year 1838, he isolated it from plant matter, determined its chemical formula and named it as "cellulose" (Payen 1838). At molecular level cellulose is a biopolymer of D-glucose units linked by β-1, 4-linkage. It primarily contains carbon (44.44 %), hydrogen (6.17 %), and oxygen (49.39 %). The chemical formula of cellulose is $(C_6H_{10}O_5)_n$ where n, called degree of polymerization, represents the number of glucose units that may range from 100 to 10,000 and even more. Although chemically the repeating unit in cellulose is glucose, structurally the repeating unit is disaccharide cellobiose, as each glucose residue is located 180˚ relative to its neighbor (Chen 2014) (Figure 1.2).

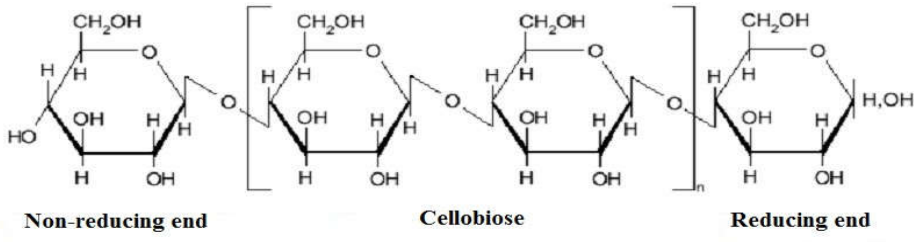


Figure 1.2: Structure of cellulose and cellobiose unit

The crystalline nature of cellulose makes it an unusual biopolymer. In the crystal, cellulose molecules are associated strongly through inter and intra molecular hydrogen-bonding and the adjacent sheets are held by Van der Waals interaction to form microfibrils with diameter of about 5-15 nm. This tight packing is responsible for the rigid structural stability of cellulose. In plant cell wall cellulose microfibrils are cross-linked together by hemicellulose whereas lignins assist and strengthen the attachment of hemicelluloses to microfibrils (Figure 1.3).

Within the microfibrils, cellulose molecule form highly ordered crystalline domains interspersed with the less ordered amorphous region. The degree of polymerization, extent of hydrogen bonding and source of cellulose, determine the amount of crystalline and amorphous components in the microfibrils. The degree of crystallinity in native cellulose is around 60-90 %. The tight packing in the crystalline structure limits the accessibility and reactivity of cellulose by affecting the rate of diffusion to not only large molecules like enzymes but also small molecules like water. Although cellulose is a homo polymer it has a heterogeneous structure consisting of crystalline and amorphous region with several surface irregularities. This heterogeneity makes the polymer capable of swelling on partial hydration which results in formation of micro-pores

and cavities and allows penetration of molecules like enzymes (Chen 2014; Madadi et al. 2017).

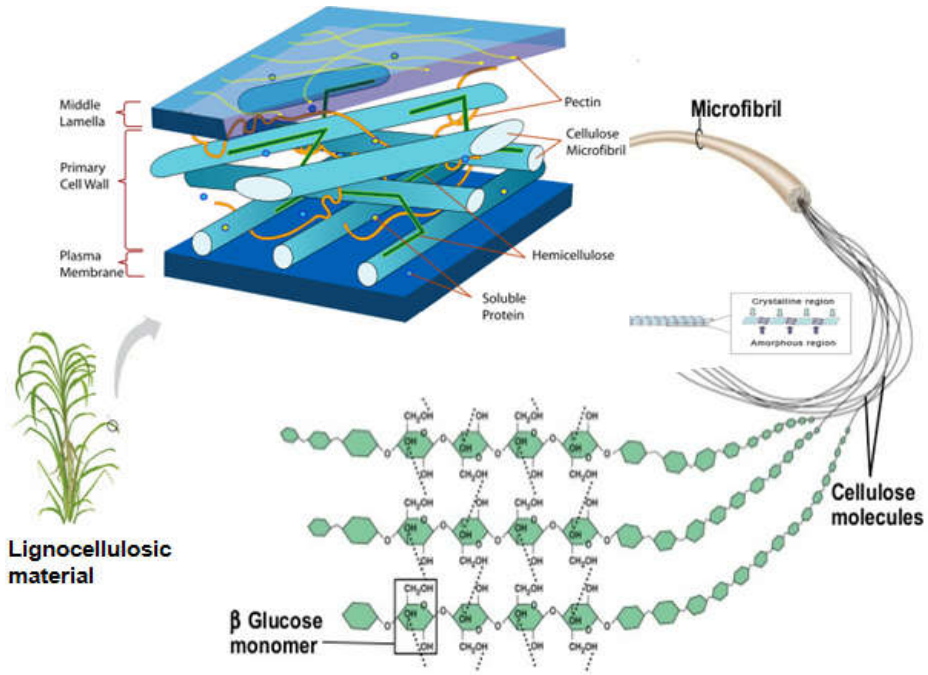


Figure 1.3: Simplified representation of arrangement of cellulose microfibrils, hemicelluloses and lignin in plant cell wall

Hemicellulose, the second most abundant biopolymer, unlike cellulose is heterogeneous in nature. It has branches with short lateral chains made up of different types of sugars mainly pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose), and uronic acids (4-O-methylglucuronic, D-glucuronic, and D-galactouronic acids). It has an amorphous structure and thus is easily hydrolyzed compared to cellulose. Hemicelluloses of plant biomass include xylan, galactan, mannan and arabinan. Xylan is the most abundant polysaccharide among hemicelluloses representing about 30-35 % of dry weight of plant cell wall. Xylan acts as a bonding agent between cellulose

and lignin. It forms covalent linkage with lignin and interacts non covalently with cellulose thereby maintaining the integrity of cellulose and protecting it against degradation from cellulase (Mussatto and Teixeira 2010) .

Lignin, a complex polymer of phenolic monomers, forms the third most abundant polymer in nature. Three main phenyl propionic alcohols are present as monomers of lignin. These include coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (p-hydroxyphenyl propanol) and sinapyl alcohol (syringyl propanol). These monomeric units are connected by aryl-aryl, alkyl-alkyl and alkyl-aryl ether bonds. The presence of lignin in plant cell wall makes it rigid, strong, impermeable and resistant to microbial attack and oxidative stress (Hendriks and Zeeman 2009).

1.2. Cellulases

Cellulose at neutral pH and in the absence of enzymes has a half-life of several million years. Microbial activity is responsible for most of the turnover of the carbon in cellulose. Microorganisms accomplish this by producing a group of enzymes named cellulases, which attack the intermolecular bonds of the cellulose biopolymer (Lynd et al. 1999). Cellulose degrading enzymes were discovered by Reese (1976). Two main strategies have been evolved by microbes to hydrolyze cellulose: discrete noncomplexed / non-aggregating cellulases and complexed / aggregating cellulases. Generally, most aerobic cellulolytic microorganisms degrade cellulose by secreting a set of individual cellulases each of which has a modular architecture including a catalytic domain joined by a flexible linker to carbohydrate-binding modules

(CBMs). The linker, connecting the two domains has repeating sequence often rich in proline, threonine, serine or glycine residues (Figure 1.4). This region plays role in catalysis by favoring interaction between the two domains (Sammond et al. 2012).

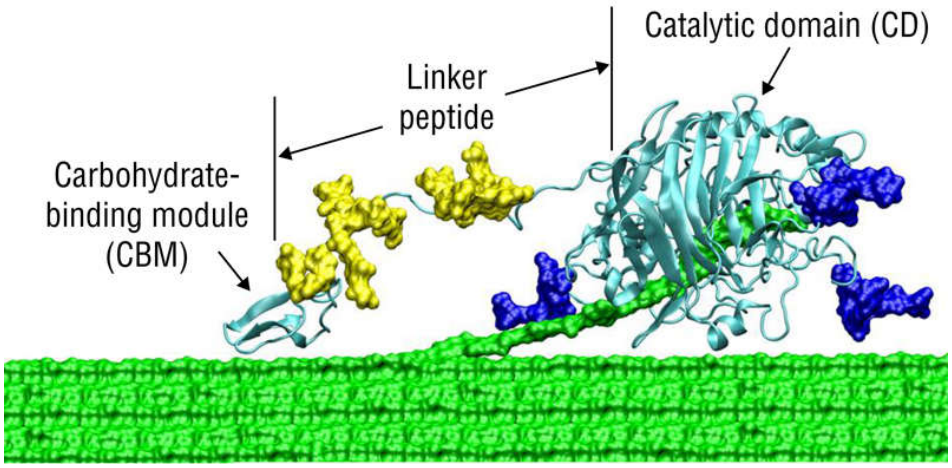


Figure 1.4: Schematic representation of modular structure of cellulase
(Credit: National Renewable Energy Laboratory)

In contrast, anaerobic microorganisms produce large cell bound multi-enzyme complexes (>1 million molecular mass) called cellulosomes (Bayer et al. 2004). Cellulosomes have a principal noncatalytic subunit called scaffoldin that integrates different enzymes and cellulosomal components into a single functional unit. The scaffoldin subunit contains substrate targeting carbohydrate-binding module (CBM) and cohesin modules, generally in tandem repeats, whereas the enzymatic subunit has a catalytic domain and a single dockerin module complimentary in specificity to the cohesin modules of the scaffoldin complex. The interaction between the scaffoldin-based cohesin modules and the enzyme-borne dockerin domains dictates the cellulosome architecture

(Figure 1.5). Some anaerobic bacteria produce both cellulosomes and free cellulases.

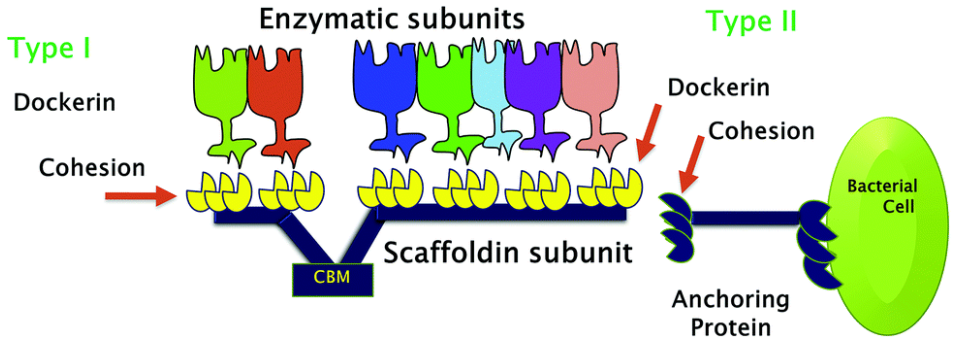


Figure 1.5: Cellulosome complex
(credit Dutta and Wu 2014)

1.3. Families of cellulase and their mechanism of action

Carbohydrates are the most diverse set of molecules in nature and thus the enzymatic machinery to synthesize, modify, and deconstruct carbohydrates is vast. Carbohydrate-Active Enzymes (CAZy) database (Bernard et al. 1998c) is a manually curated list of the primary enzyme classes known to act on carbohydrates. It describes families of structurally – related catalytic and carbohydrate binding modules of enzymes that degrade, modify or create glycosidic bonds. The protein classes covered under CAZy database are glycoside hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), auxiliary activities (AAs) and carbohydrate - binding modules (CBMs).

Cellulases belong to GHs, which catalyze the hydrolysis of glycoside bond that link two or more carbohydrate molecules or a carbohydrate and non-carbohydrate molecule. The glycoside hydrolases

are grouped into different families based on the amino acid sequence similarity. The classification system was introduced by Bernard Henrissat in 1991 (Henrissat 1991). An up-to-date version of the classification of GH (EC 3.2.1) families is maintained by the CAZy database. Currently glycoside hydrolases are divided into 145 families designated as GH1-GH145 (Bernard et al. 1998b). Enzymes with characterized cellulolytic activities belong mainly to the families GH1, GH3, GH5, GH6, GH8, GH9, GH12, GH45, GH48, GH51 and GH74 (Brumm 2014). The GH families are further divided into 14 clans (GH-A to GH- N) based on protein tertiary structure. Each clan has two or more families that have the same three-dimensional fold in the catalytic module. The sequence similarity between the families within a clan may however differ. This proves that protein structure is better conserved in evolution than the amino acid sequence (MacGregor 2005).

All known glycoside hydrolases employ one of the two mechanisms for hydrolysis: either retaining or inverting hydrolysis, as first described by Koshland (1953). The glycosidic bonds are cleaved by acid-base catalysis, mainly involving two catalytic residues in the enzyme: a general acid (proton donor) and a nucleophile/base (Davies and Henrissat 1995). Depending on the spatial position hydrolysis can occur either by retention or inversion of the anomeric configuration. In retaining cellulases a net retention of anomeric C bearing the target glucosidic bond is achieved by a two step double-displacement mechanism. In contrast, for inverting cellulases the anomeric C invert its configuration after a single nucleophilic displacement hydrolysis (Vocadlo and Davies 2008).

Often the catalytic function for carbohydrates is associated with binding function which is classified into CBMs in the CAZy database. CBMs have three general functions (i) proximity effect that is; CBMs concentrate enzymes on the surface of substrate through their sugar binding activity, maintaining the enzyme in proximity with the substrate leading to increased degradation of the polysaccharide (ii) substrate targeting/selectivity function and (iii) a disruptive function, which disrupts the non-hydrolytic crystalline substrate (Boraston et al. 2004). CBM may be located in either the N or C-terminus of the catalytic module, but its location is not related to its function (Yang et al. 2013).

To date 81 families of CBM are known (Bernard et al. 1998a). Boraston et al. (2004) manually classified these families into seven fold families based on the structure and confirmed structural similarity using DALI search. But grouping of CBM into fold families based on the conservation of protein fold are not predictive of function. Sufficient diversity exists among the fold families such that functional elements, either specific amino acids or binding-site topographies, are not conserved. Thus prediction of ligand specificity based on possession of particular fold must be considered with caution. To overcome these limitations another classification of CBMs based on the structural and functional similarities have been proposed. In this the protein modules are grouped into three types: ‘surface-binding’ CBMs (Type A), ‘glycan-chain-binding’ CBMs (Type B), and ‘small sugar-binding’ CBMs (Type C). Cellulases have type B CBMs that bind to cellulose (Boraston et al. 2004).

1.4. Types of cellulases and mode of action

Microorganisms utilize insoluble cellulose by secreting extracellular cellulases that are either free or cell bound. Components of the cellulase system have been classified based on mode of action and structural properties (Henrissat et al. 1998). Three major types of enzymatic activities are found: endoglucanases, exoglucanases and β-glucosidases. These enzymes act synergistically to bring about the complete hydrolysis of cellulose to glucose (Figure 1.6).

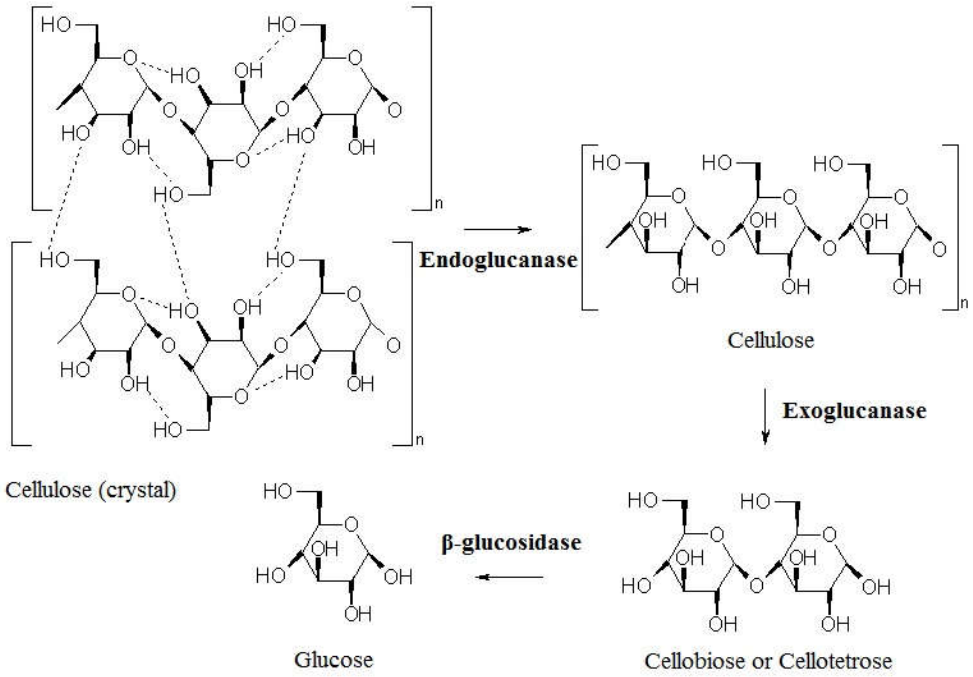


Figure 1.6: Types of cellulase and mode of cellulase action

1.4.1. *Endoglucanase*

Endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4) or CMCase cuts β-1, 4-glucosidic bonds randomly at internal amorphous sites generating oligosaccharides of various lengths and consequently new chain ends. The catalytic modules of most endoglucanases have a cleft/grove-shaped active site which allows it to bind and cleave the cellulose chains to yield glucose, soluble cellodextrins or insoluble cellulose fragment. However, some endoglucanases have the ability to hydrolyze crystalline cellulose and generate the major products as cellobiose or longer cellodextrins. These processive endoglucanases can substitute for cellobiohydrolases in cellulase system and thus play a major role in the degradation of cellulose (Wu et al. 2018).

1.4.2. *Exoglucanase*

Exoglucanases are enzymes that cleave the accessible ends of cellulose molecules to liberate glucose and cellobiose. There are two types of exoglucanases: 1,4-β-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and 1,4-β-D-glucancellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91). Both these exoglucanases act in a processive manner, on reducing or non reducing ends of cellulose chains and liberate glucose in case of cellodextrinases and cellobiose in case of cellobiohydrolase as major product. The catalytic module of all exoglucanase is a tunnel structure which is formed by two surface loops. The active site inside the tunnel enables the enzyme to hydrolyze cellulose in a unique “processive” manner (Vocadlo and Davies 2008). Exoglucanases have low activity on CMC and do not decrease its

viscosity but it effectively works on microcrystalline cellulose (Yang et al. 2013).

1.4.3. *β-glucosidases*

β-glucosidases or β-glucoside glucohydrolases (EC3.2.1.21) completes the degradation of cellulose by hydrolyzing soluble cellodextrin and cellobiose formed by previous enzymes to liberate glucose. It does not show any activity towards insoluble cellulose. The pocket-shaped active site allows it to bind to the non-reducing glucose units and cleave glucose off from the oligosaccharides. The enzyme degrades cellobiose which is thought to be an inhibitor of cellobiohydrolase and endoglucanase (Yang et al. 2013).

The enzymes of cellulase system show synergism; that is their collective activity is greater than the sum of their individual activities. Four major synergisms have been reported (a) endo-exo synergy (between endoglucanases and exoglucanases), (b) exo-exo synergy (between exoglucanases processing from the reducing and non-reducing ends of cellulose chains) (c) exoglucanase-β-glucosidase synergy (removes cellobiose (and cellodextrins) which are end products of the first two enzymes), and (d) CD-CBM synergy (intramolecular synergy between catalytic domains and carbohydrate binding modules) (Lynd et al. 2002).

1.5. Cellulolytic microorganisms

Cellulases are inducible enzymes produced by bacteria, fungi, protozoans, plants and animals (Yang et al. 2013). However, enzymes of microbial origin are more widespread due to their broad biochemical

diversity, feasibility of mass culture, and high degree of stability under extreme conditions. Moreover, they allow easy genetic manipulation. Presently most of the commercially available cellulases are obtained from fungi mainly *Aspergillus, Humicola, Trichoderma,* and *Penicillium*. Other cellulolytic fungi are represented by species of *Alternaria, Acremonium, Coniothyrium, Chaetomium, Cladosporium, Ceratocystis, Fusarium, Myrothecium, Memnoniella, Phoma, Stachybotrys, Thievlavia,* etc. However, bacterial cellulases are gaining attention because of their high natural diversity, higher growth rate, easier product recovery and ability to produce enzymes that withstand harsh environmental conditions (Maki et al. 2009). The cellulolytic potentials of bacteria belonging to different genus such as *Acetivibrio, Bacillus, Bacteroides, Cellulomonas, Clostridium, Erwinia, Ruminococcus* and *Thermomonospora* have been well studied (Robson and Chambliss 1989). The microbes commercially exploited for cellulase production are *T.reesei, H.insolens, A. niger, Thermomonospora fusca, Bacillus* sp. and few others (Sukumaran et al. 2005).

1.6. Regulation of cellulase production

Cellulases are inducible enzymes and its production is finely controlled by induction and repression mechanism. Cellulase production is induced in the presence of cellulosic substrate and repressed when easily utilizable sugars are available. Sophorose and cellobiose are natural inducers of cellulase production. The hydrolysis product of cellulase, mainly cellobiose which is produced by basal level expression of the enzymes, acts as the inducer. Moreover, the transglycosylation activity of β-glucosidase generates sophorose, which is also a strong inducer of

cellulase enzymes (Mandels et al. 1962). Catabolite repression of cellulase genes occurs in the presence of glucose and is thought to be regulated by cAMP. But at low concentrations, glucose is found to induce cellulase production (Lynd et al. 2002; Sukumaran et al. 2005). Cellulase enzymes are also prone to end product inhibition. Accumulation of cellobiose and glucose which are major end products of hydrolysis inhibits cellulases and causes reduction in glucose production. Among these cellobiose is a strong inhibitor of endoglucanase whereas β-glucosidase is inhibited by high concentrations of glucose (Holtzapple et al. 1990).

1.7. Cellulase production by fermentation

Fermentation is the intentional use of microorganisms for the production of various valuable commercial products. This is the basic technique for production of various enzymes. Both fungi and bacteria produce an array of enzymes when fermented on suitable substrate. The first enzyme produced industrially was taka-diastase (a fungal amylase) in 1896, in United States. Fermentation process for enzyme production can be classified into (i) submerged fermentation (SmF) and (ii) solid-state fermentation (SSF). The main difference between the two is that free flowing water is present in SmF whereas it is low or almost absent in SSF. Submerged fermentation technique is more suited for microorganisms like bacteria that require high moisture content whereas SSF is preferred by fungi and microorganisms that require less moisture content (Subramaniyam and Vimala 2012). Majority of the studies on microbial production of cellulase uses SmF technology and the most widely studied organism is *Trichoderma reesei.* Although in nature the growth and

utilization of cellulose by cellolulytic microorganisms resembles solid state fermentation than liquid culture, the advantage of better handling and monitoring of the process is associated with SmF (Sukumaran et al. 2005). More than 75 % of industrial enzymes including cellulases are produced using SmF. The main reason is that SmF supports the use of genetically modified organisms to a greater extent than SSF. Currently cellulases are obtained from genetically modified strain of *Hypocrea jecorina* (anamorph *Trichoderma reesei*) by SmF. Another reason for the wide use of SmF is the lack of complicated equipments and accessories required for production of various enzymes by SSF. SmF is mainly used for production of secondary metabolites that are used in liquid form. The easier purification of the product gives this technique an additional advantage (Subramaniyam and Vimala 2012). SSF technique had been gaining attention because it is cost effective, not only for enzyme production but also for the bioconversion of lignocellulosic waste using cellulolytic microorganism. The studies conducted by Tengerdy showed a 10-fold reduction in production cost in SSF compared to SmF (Tengerdy 1996). Although there are reports on cellulase production by SSF, large scale production of cellulase is by the proven technology of SmF (Sukumaran et al. 2005).

1.8. Applications of cellulase

Cellulases were initially investigated in about 1970s, during the gasoline shortage, for the bioconversion of biomass into sugars which could be fermented to ethanol and used as an alternative fuel. This paved the way to research in industrial applications of the enzyme in detergents, food, animal feed, paper and pulp, and textile industry. The first large

scale industrial use of cellulase was in mid 1980s in the stonewashing denim and as an additive of animal feed. But, due to depletion of fossil fuels and increasing demand for biofuels, there is renewal of interest in use of cellulase for bioconversion of lignocellulosic biomasses. However, in other industries the use of cellulase has become indispensible.

1.8.1. *Bioethanol production*

The limited supply and environmental pollution caused due to fossil fuels has forced the world economy to focus on biofuels especially bioethanol from renewable resources. Biofuels are expected to replace 20 % of the fossil fuel consumption by 2020. Thus the most important application of cellulase which is currently being investigated is in bioconversion of lignocellulosic wastes into biofuels. Cellulases convert the cellulosic biomass into glucose and other fermentable sugars which in turn is used as substrate for production of bioethanol. The production of bioethanol is a multi-step process involving preteatment of biomass to remove lignin and hemicellulose fraction, cellulase treatment at 50 ^{o}C to hydrolyze cellulosic residues and generate fermentable sugars and finally use of fermentative organisms for the production of alcohol from the hydrolyzed cellulosic material (Duff and Murray 1996). Overall, lignocellulosic biomass is an attractive substrate for the production of biofuels. Thus lignocellulosic wastes generated from forests, agricultural and agro-industries can be judiciously utilized, leading to a two in one remedy for environmental pollution and energy crisis.

1.8.2. *Paper and pulp industry*

The use of cellulase in paper and pulp industry has increased drastically over the past few decades. The enzymes are mainly used for biomechanical pulping and for deinking of waste papers. Pulping using enzymes has the advantage that it is ecofriendly; it improves the efficiency of the process and also enhances the mechanical and physical strength of final paper product. Enzymatic deinking of waste paper especially using cellulases and hemicellulases enhances the quality and brightness of recycled paper (Ibarra et al. 2012). Cellulases have also been used for improving the drainage and runnablility of paper mills by dissolving clogged fiber residues. Cellulase preparations are also being used for production of biodegradable cardboard, sanitary paper and paper towels, and also used to remove adhered paper (Kuhad et al. 2011).

1.8.3. *Textile industry*

Cellulases have been successfully used in textile industries for improving the appearance and softness of cellulose based textiles. They are mainly used for biopolishing of cotton cloths and biostoning of denim jeans. During the process of biopolishing, cellulase acts by hydrolyzing the small protrusions of the fibres from the surface of cotton clothes, thereby removing the fussiness of the surface and giving the cloths a smooth and glossy appearance. It also improves the color brightness, hydrophilicity and moisture absorbance (Bhat 2000). Cellulases have replaced the traditionally used pumice stones for biostoning of denim garments for producing softness and faded look of jeans. During biostoning, cellulases hydrolyze the small fibre protrusions from the yarn

surface thereby loosening the indigo dye, which is easily removed by mechanical abrasion in the wash cycle. The advantage of biostoning over use of pumice stone is less damage to fibers, increased productivity of the machines, less work-intensive and environment friendly (Belghith et al. 2001). *Humicola insolens* and *Trichoderma* are commonly used for biostoning of jeans (Sukumaran et al. 2005). Cellulases, when used as additives in household laundry detergents, improves the fabric softness, brightness and also helps in removal of dirt particles trapped within the microfibril network.

1.8.4. *Detergent industry*

Recently the detergents used in industries have a cocktail of enzymes usually cellulase, lipase and protease. Cellulase preparations can modify the cellulose fibrils and improve color brightness, feel, and dirt removal from the cotton blend garments. Alkaline cellulases, in the presence of conventional detergent ingredients, have the potential of removing soil in the interfibril spaces by selective contact with cellulose within the interior of fibers. Liquid laundry detergents often contain anionic or nonionic surfactant, citric acid or a water-soluble salt, protease, cellulase, and a mixture of propanediol and boric acid or its derivative. The detergents are prepared by adding diol and boric acid before adding acid or salt to the composition. These ingredients and the order of addition can improve the stability of cellulases (Boyer and Farwick 1993). Cellulase preparations that are active under mild alkaline conditions and elevated temperatures are commonly added to detergents and washing powders.

1.8.5. *Food and animal feed industry*

Cellulases have wide application in food industry. It forms a part of the macerating enzyme complex (cellulase, xylanase and pectinase), used for the extraction and clarification of fruits and vegetable juices, oils, nectars and purees (Ajayi et al. 2015). Macerating enzymes increase the yield of juice and process performance without additional capital investment (Bhat 2000). Cellulase in combination with other cell wall degrading enzymes like hemicellulase, ligninase, pectinase etc. can enhance the texture, flavor, and aroma of citrus fruits by reducing the bitterness. Enzyme mixtures containing pectinases, cellulases, and hemicellulases are also used for improved extraction of olive oil (Kuhad et al. 2011). Cellulases are also used in extraction of carotenoids in production of food coloring agent (Çinar 2005). Cellulases find application in production of alcoholic beverages (beer and wine) due to its potential to release simple sugars from cellulosic materials. These enzymes improve the quality and yield of fermented foods (Bamforth 2009).

Cellulases have potential application in animal feed industry consumed by poultry, pig ruminants as well as pet and fish farming (Karmakar and Ray 2011a). Enzyme preparations including hemicellulase, pectinase and cellulase are used to improve the nutritive quality of forages. Use of cellulases in feed processing is known to improve feed digestibility and animal performance (Graham and Balnave 2007).

1.8.6. *Other applications*

Apart from the common applications cellulases are used in formulations for removal of industrial slime (Wiatr 1990), to generate protoplast for genetic manipulation (Liu and Zhu 2000) and for production of antibacterial chitooligossacharide which could be used in food preservation (Tsai et al. 2000), immune-modulation (Wu and Tsai 2004) and as a anti tumor agent (Qin 2004). Besides this cellulases have potential applications in agriculture and pharmaceutical industries. Enzyme preparation containing cellulase, hemicellulase and pectinase are used for enhancing plant growth of crops and controlling plant diseases (Han and He 2010). In pharmaceutical industries cellulases are used in digestive capsules, like digestin for optimal digestion.

1.9. Cellulase market scenario

The demand for cellulase is constantly rising due to its applications in textile, paper, detergent and other industries. Although there are many companies involved in cellulase production, globally, there are only two main companies, which have been engaged in active research for developing cellulase for biomass conversion. “Genencor” and “Novozymes” have played major role in reducing the cost of cellulase enzyme by adopting new technologies (Table 1.1). Amano Enzyme Inc., Japan and MAP’s India in India are other enzyme companies actively involved in cellulase production. Although a number of enzyme producing companies are involved in cellulase production and marketing, only few of them have developed cellulase for conversion of biomass.

Table 1.1: Major cellulases developed by Genencor and Novozymes

Year	Enzymes	Features	Reference
		Genencor	
2007	Accellerase®1000	• Enzyme for cellulosic ethanol	
2009	Accellerase®1500	• Produced using a genetically modified strain of *T. reesei*. • is a cellulase complex (exoglucanase, endoglucanase, hemicellulase and β -glucosidase) • Cost effective and efficient for bioethanol industries (contains higher levels of β-glucosidase activity than other enzymes, to ensure almost complete conversion of cellobiose to glucose)	Li (2009)
2009	Accellerase®XP	• has accessory xylanase • enhances both xylan and glucan conversion	
	Accellerase®XC	• is an accessory xylanase/cellulase enzyme complex • has hemicellulase and cellulase activities	
	Accellerase® BG	• is an accessory β -glucosidase enzyme	
2010	Accellerase®Duet	• improved overall hemicellulase activity and higher biofuel yield than its predecessor-Accellerase®1500	Wyers (2010)
2011	Accellerase®TRIO	• a cocktail of enzymes that breakdown the glucan (C6) and xylan (C5) • has the capacity to unlock 70 to 90 % of sugars from biomass • When paired with an effective pre-treatment, it delivers complete liquefaction in 24 h at economically viable dosing	Voegele (2011)
		Novozymes	
2008	Cellusoft®AP and Cellusoft®CR	• bioblasting in textile mills	Novozymes (2008)
	Carezyme® and Celluclean	• laundry in detergent	
	Denimax® 601l	• for stonewash industry at low temperature	
2010	Cellic® CTec2 and HTec2	• biomass conversion • When both the enzymes are used together they can efficiently work with wide variety of pretreated feedstocks, for the conversion of the carbohydrates into simple sugars	Novozymes (2010)

1.10. Objectives of current study

In order to meet the future challenges, innovative bioprocesses for the production of new generation of enzymes is needed. The bottleneck of cellulase research is the cost of enzyme. Major research is thus directed towards identification of efficient cellulase producers, developing processes for cost effective production of cellulases, genetic manipulation of existing organisms, protein engineering to improve properties like specific activities, process tolerance and thermal stability, designing enzyme cocktail and developing novel strategies for efficient conversion of biomass. In the light of these facts present study was undertaken with the following research objectives:

1. Isolation, screening and identification of cellulolytic bacteria.

2. Production and statistical optimization of β-1, 4 endoglucanase by *Bacillus subtilis* MU S1.

3. Exploitation of agro waste as low cost substrate for endoglucanase production and optimization of agro-waste medium.

4. Cloning, expression and characterization of endoglucanase gene from *Bacillus subtilis* MU S1 in *Escherichia coli*.

5. Application of recombinant endoglucanase in saccharification of agro-wastes.

SREENA C. P. “IMPROVEMENT OF BIOCATALYTIC EFFICACY OF ENDOGLUCANASE FROM INDIGENOUS STRAIN, BACILLUS SUBTILIS MU S1 AND ITS APPLICATION IN SACCHARIFICATION OF AGRO-WASTES”. THESIS. DEPARTMENT OF LIFE SCIENCES, UNIVERSITY OF CALICUT, 2018.

2.1. INTRODUCTION

Cellulose, one of the major constituent of the cell wall of plants is the most abundant and renewable biopolymer on earth. The complete degradation of cellulose involves the synergistic action of three hydrolases. First, endo-β-1, 4-glucanases [EC 3. 2. 1. 4] nicks the internal cellulose chain, after which exo-β-1, 4-glucanases or cellobiohydrolases [EC 3. 2. 1. 91] attacks the ends of the crystalline structure and releases cellobiose processively or nonprocessively and finally, β-1, 4-glucosidases [EC 3. 2. 1. 21] cuts cellobiose and cellooligosaccharide to produce glucose (Bhat and Bhat 1997). Cellulases are currently the third most important enzymes in the market due to their wide range of applications. However, the demand for cellulases is growing rapidly because of its role in production of transportation fuel, and this demand is the driving force for cellulase research. Significant research therefore has been directed toward identification of novel enzyme producers and process conditions besides those aimed at modification of existing organisms (Mohanram et al. 2013; Liang et al. 2014; Chuan Wei et al. 2015). Novel microbes secreting highly component enzymes can be obtained by bioprospecting enzyme producers from less studied environments.

Cellulolytic microorganisms are ubiquitous in nature and have been isolated from various sources like forest soil (Woo et al. 2014), hot springs (Tamariz-Angeles et al. 2014), composts (Fathallh Eida et al. 2012), dairy manure soil (Devi and Kumar 2012), cowdung (Bai et al. 2012), wild and domestic ruminants (Sahu et al. 2004), GI tract of giant African snail (Pawar et al. 2015), termites (Sreena et al. 2015), and

municipal solid wastes (Prabesh et al. 2016). As soil and the litter horizon contain the largest pool of organic C in the terrestrial biosphere, extracellular enzyme producers thrive in these environments (Magnani et al. 2007). However, due to the high heterogeneity and richness, our knowledge on the soil microbes remains limited. Only 1 % of soil microorganisms have been identified, leaving behind a staggering 99 % yet to be discovered. Forest ecosystem is the most complex and heterogeneous terrestrial environment, characterized by a wealthy biological diversity with millions of plants, animals and microorganisms in their natural environment (Srividya et al. 2008). It is estimated that 30 g of forest soil contains over half a million species. Kerala forests form a part of Western Ghats which are biodiversity hotspots and potential reservoir of indigenous microorganism but little studied and less exploited.

Cellulases are inducible enzymes obtained from fungi, actinomycetes and bacteria during their growth on cellulosic material. Most studies have given emphasis on fungi for cellulase production because they produce copious amount of enzyme that are less complex than bacterial enzyme, thus allowing easy extraction and purification. However, the past decades have seen a shift towards isolation and characterization of novel cellulase from bacteria. The reason for such a change is the higher growth rate of bacteria, increased function and synergy of multienzyme complexes, and low feedback inhibition of bacterial cellulase. Moreover the ability of bacteria to survive in extreme conditions results in the production of more effective biocatalysts stable under harsh conditions required for various industrial applications (Maki

et al. 2009). Considering the importance of bacterial cellulases an attempt was made to screen different samples and isolate efficient cellulolytic bacteria.

2.2. OBJECTIVES

The main objectives of this chapter were to isolate, screen and identify efficient cellulolytic bacteria from different samples. The ability of the selected strain to secrete the different cellulolytic enzymes was investigated. The hydrolytic repertoire of the selected strain was also scrutinized by plate assays, using distinct substrates.

2.3. REVIEW OF LITERATURE

Cellulases are the key enzymes involved in the degradation of cellulose, the most abundant organic matter on earth. Although cellulases used in industrial applications are obtained from fungi, bacteria have also been considered as robust and versatile enzyme producers. Bacterial cellulases have recently gained importance as a potential source for development of commercial process because of high growth rate, wide genetic variability and adaptability, and high amenability to genetic manipulation.

2.3.1. Soil as source of cellulolytic bacteria

Soil has a wide array of aerobic cellulolytic bacteria, belonging to various phyla including *Firmicutes*, *Actinobacteria*, *Bacteroidetes,* and *Proteobacteria.* Among them the members of the genus *Bacillus*, *Cellulomonas*, *Streptomyces*, *Cytophaga*, *Cellovibrio,* and *Pseudomonas* have been characterized (Lynd et al. 2002). According to the studies of

Hatami et al. (2008) total number of bacteria and the number and percentage of cellulolytic bacteria in forest soil samples are more than in farming soil. Though soil is the most widely exploited source for isolation of celluloytic bacteria, less importance has been given to forest soil. Cellulolytic bacteria belonging to the genus *Bacillus* (Behera et al. 2014; Fatema and Manchur 2015), *Pseudomonas*, *Xanthomonas, Brucella , Micrococcus* (Behera et al. 2014), *Rhodococcus*, *Stentrophomonas*, *Variovorax*, *Serratia*, *Janthinobacterium* (Avellaneda-Torres et al. 2014) and *Methylobacterium* (Jayashree et al. 2011) have been isolated from soils of different forests.

Recently, cellulase research in Kerala, has led to isolation of different fungal, actinomycete and bacterial strains. Cellulase degrading fungi have been isolated from soil samples from Neyyar wild life sanctuary (Balakrishnan et al. 2015; Parambath et al. 2016) and mangrove soils (Gilna and Khaleel 2011; Nathan et al. 2014); actinomycetes were isolated from southern western ghats (George et al. 2010). Bacterial strains were obtained from wood-yards on Kallai river bank (Sreedevi et al. 2013) and industrial and agricultural areas (Vimal et al. 2016). However, in search of novel cellulolytic organisms, different areas need to be explored.

Isolation of novel microbial species from extreme and unexplored habitats is one of the competent strategies for the mining of potential microbial compounds. Western Ghats are considered as biodiversity hotspots and have served as a source of potent microorganisms that could be exploited for various industrial and medicinal applications. Nampoothiri et al. (2013) in their review have given a detailed account of

the microorganism isolated from Western Ghats of India and their industrial applications. Over the past few years, the Western Ghats of Kerala have been investigated for novel and industrially relevant microorganisms. Dastager et al. (2010b) isolated a novel bacterium *Pontibacter niistensis* NII-0905(T) from soil sample of Western Ghats of Kerala. Table 2.1 gives a list of microorganisms isolated from different areas in Western Ghats of Kerala and the industrially important biomolecules produced.

2.3.2. Isolation, screening and identification of cellulolytic bacteria

In the past, screening strategies using soil samples were highly empirical requiring extensive experimentations. But with the advent of enrichment culture, screening and selection have become less complex. Martinus Beijerinck and Sergei Winogradsky are credited with the development of enrichment culture technique. Enrichment culture, containing cellulosic substrate as the sole source of carbon, has been widely used by researchers for isolation of cellulase producers. The different cellulosic substrates used include carboxymethyl cellulose (CMC), filter paper strips, powdered cellulose and amorphous phosphoric acid treated swollen cellulose (Mohagheghi et al. 1986).

Screening for extracellular cellulase production by microbes is usually done using plate assay. Since crystalline cellulose is degraded at a slow rate, easily degradable soluble cellulose derivatives like CMC are used for most of the assays (Yeoh et al. 1985).

Table 2.1: List of microorganisms isolated from Western Ghats of Kerala and their industrial applications

Microorganism	Application	Area	Reference
B. thuringiensis	Insecticidal activity	Kottayam, Kozhikode and Ernakulam	Neema et al. (2009)
Exiguobacterium NII-0906	Plant growth promoting rhizobacteria	Rhizosphere soil	Dastager et al. (2010a)
Streptomycetes	Enzyme production (Cellulase, pectinase, xylanase, amylase and protease), antibacterial activity	Kuruva island and Pookot lake, Wayanad	Hamedani et al. (2012)
Actinomycetes (Streptomyces, Nocardia, Micromonospora, Pseudonocardia, Streptosporangium, Nocardiopsis and *Saccharomonospora)*	Antibacterial activity	Eravikulam National park, Idukki	Varghese et al. (2012)
Actinomycetes (Streptomyces, Nocardia, Micromonospora, Pseudonocardia, Streptosporangium, and *Nocardiopsis)*	Antibacterial activity	Shendurney Wildlife Sanctuary, Kollam	Varghese et al. (2014)
Paenibacillus elgii NIISTB523	Antimicrobial activity	Wayanad	Kumar et al. (2015)
Streptomyces, Pseudomonas, Bacillus and *Trichoderma*	Antagonistic activity	Rhizosphere soil	Ramkumar et al. (2015)
Streptomyces sp., *Bacillus* sp., *Paenibacillus* sp. , *Lysinobacillus* sp.	Cellulase, xylanase	Arikan Para, Puchi Para, Neelikal	Amore et al. (2015)
Bacillus sp.	Antifungal plant probiotic agent	Rhizosphere soil	Jimtha John et al. (2016)
Streptomyces sp. SFA5	Antimicrobial activity	Sabarimala forest ecosystem	Ponnuswamy et al. (2016)
Aspergillus terreus KMBF1501	Pigment production	Idukki	Akilandeswari and Pradeep (2017)

Solid media containing CMC to detect cellulase producing microorganisms was developed by Hankin and Anagnostakis. They used 1 % aqueous hexadecyltrimethyl-ammonium bromide for flooding the plates to observe the zone produced by hydrolysis of CMC (Hankin and Anagnostakis 1977). Over the years different dyes have been used by researchers for the detection of zone. These include Congo red (Teather and Wood 1982), remazol brilliant blue, phenol red, tryphan blue (Yoon et al. 2007), Gram's iodine (Kasana et al. 2008), coomassie brilliant blue and safranin (Gohel et al. 2014).

Congo red staining, followed by destaining with 1M NaCl is the most widely used method. It is based on the interaction of Congo red with intact β-(l-4)-D-glucans in CMC. It is cost efficient, simple, and convenient method; moreover it gives a zone diameter of hydrolysis of CMC that truly corresponds to the results obtained with the dinitrosalicyclic acid reagent method. This method is time consuming and takes about 30- 40 min. According to Kasana et al. (2008) this limitation can be overcome by use of Gram's iodine for detection of zone. Gram's iodine formed a bluish-black complex with cellulose but not with hydrolyzed cellulose, giving a sharp and distinct zone around the cellulase-producing microbial colonies within 3 to 5 min. But recently Meddeb-Mouelhi et al. (2014) found that Gram's iodine may lead to identification of false positives. Amylase activity and the hydrolysis of contaminating starch contained in the agar may be responsible for the lack of reliability of this test (Zitomer and Eveleigh 1987). Thus Congo red test is still the most reliable as it avoids such pitfalls.

After screening the isolated bacteria are subjected to different tests for the purpose of identification and classification. Traditional method of identification and classification rely on phenotypic identification using Gram's staining, culture and biochemical methods. Bergey's Manual of Determinative Bacteriology, is used as a standard reference for identification of bacteria upto genus level (Bergey et al. 1974). But these methods suffer major disadvantages and fail to produce accurate results. Moreover, because of the limited number of biochemical and phenotypic tests available, characterization of proposed species was inadequate and imprecise.

Over the first half of the 20^{th} century, a number of approaches to the identification and classification of bacteria were introduced.16S rRNA gene sequencing is still the most widely used technique. It has become a routine technique for identification and classification of bacteria in most microbiological laboratories. The 16S rRNA gene provides genus identification in > 90 % of cases, and identification of 65–83 % of these at the species level. The rRNA sequences were shown to be a very useful molecular marker for phylogenetic analyses. Molecular phylogeny increasingly supports the understanding of organismal relationships and provides the basis for the classification of microorganisms according to their natural affiliations (Drancourt et al. 2000).

Over the years, a variety of sources have been explored by researches for isolation of cellulolytic bacteria. Although bacteria belonging to different genera like *Bacillus*, *Clostridium*, *Cellulomonas*, *Paenibacillus* have been characterized (Table 2.2), studies are still progressing for isolation of highly efficient cellulase producers.

Table 2.2: List of cellulolytic bacteria isolated from various sources and the method used for isolation and screening

Strain	Sample	Substrate used for isolation	Detection method	Reference
Cellulomonas sp.	Rotting sugarcane stalk and adjacent soil	Filter paper	Visual degradation of filter paper	Han and Srinivasan (1968)
Cellulomonas fermentans sp.*nov.*	Municipal dumping ground	ball-milled MN300 cellulose	Clear zone	Bagnara et al. (1985)
Acidothermus cellulolyticus llBT	acidic hot springs	filter paper, Powdered Cellulose,CMC, amorphous Phosphoric acid- treated swollen cellulose	Clear zone	Mohagheghi et al. (1986)
Bacillus brevis VS-1	soil	Glucose	Congo red and 1N acetic acid	Singh and Kumar (1998)
Cellulomonas gelida, Cellulomonas biazotea Cellulomonas cellulans, Flavobacterium johnsoniae	soil	natural cellulosic fibres	Clear zone	Lednicka et al. (2000)
Anoxybacillus flavithermus EFP1, *Geobacillus thermodenitrificans* EFP2, *Geobacillus stearotherm Ophilus* EFP3	water and soil samples, Egyptian hot spring	CMC	Congo red and NaCl	Ibrahim and El-diwany (2007)

Bacillus cereus strain Razmin A, *Enterobacter aerogenes strain Razmin* B, *Enterobacter cloacae strain Razmin* C, *Chryseobacterium kwangyangense* strain *Cb* and *Acinetobacter* strain *Raminalimon*	gut of the termite *Coptotermes curvignathus* (Holmgren)	CMC	Clear zone	Ramin et al. (2009)
Cellulomonas sp. YJ5	soil in northern Taiwan	CMC	Clear zone	Yin et al. (2010b)
Cornybacterium lipophiloflavum	decaying vegetables	CMC	Congo red and NaCl	Sakthivel et al. (2010)
Geobacillus pallidus EB compost 1	Compost samples	CMC	Congo red and NaCl	Baharuddin et al. (2010)
Cellulomonas sp. *strain* TSU-03	soil	CMC	Congo red	Sangkharak et al. (2011)
Sphingomonas sp., *Pseudomonas* sp.M1, *Achromobacter* sp., *Pseudomonas*sp.M2, and *Stenotrophomonas* sp	soil samples	CMC	Clear zone	Chen et al. (2011)
Bacillus subtilis	Cow dung	CMC	Congo red and NaCl	Bai et al. (2012)
Cellulomonas ASN2	soil	CMC	Congo red and NaCl	Irfan et al. (2012)
Pseudomonas sp. *Staphylococcus* sp. *Acinetobacter* sp. *Bacillus* sp., *Salmonella* sp.	Gut of Xylophagous Termite *Microcerotermes diversus*	CMC	Congo red	Pourramezan et al. (2012)

Variovorax sp. CB1-2-A-5 *Paenibacillus* sp. VG-4-A-2, VG-4-A-3, *Staphylococcus* sp. CB1-2-A-4	forest soils	CMC	Congo red and NaCl	Ghio et al. (2012)
Bacillus amyloliquefaciens strain SS35	Rhinoceros Dung	CMC	Congo red and NaCl	Singh et al. (2013)
Bacillus licheniformis JK7	Rumen of a Native Korean Goat	CMC	Clear zone	Seo et al. (2013)
Clostridium thermocellum strains, CS7 and CS8	compost sample	milled filter paper	Clear halo	Lv and Yu (2013)
B. velesensis P3-1 and P4-6 *Paenibacillus* strain S10-4	soil samples	Powdered Cellulose	Congo red and NaCl	Akaracharanya et al. (2014)
Bacillus licheniformis and *Cohnella laeviribosi*	Combined sediment and water samples from hot spring, Peru	filter paper, glucose	Congo red and NaCl	Tamariz-Angeles et al. (2014)
Fibrobacter Succinogenes NBG	rumen of inner Mongolia sheep	CMC	Clear zone	Liu et al. (2014)
Bacillus subtilis BY-2	Tibetan pig's intestine	CMC	Congo red and NaCl	Yang et al. (2014)
Pantoea dispersa A3	spoiled apple fruits	CMC	Congo red and NaCl	Atala Mohammed L et al. (2014)
Klebsiella sp. PRW-1	soil samples	CMC	Congo red	Waghmare et al. (2014)
Clostridium sp., *Bacillus* sp.	coffee exocarp	filter paper	Congo red	Bui (2014)
Isoptericola variabilis sp. IDAH9	water and soil samples from hot springs	CMC	Congo red and NaCl	Azizi et al. (2015)

Stenotrophomonas sp. MY6 *Bacillus cereus* FY2	fresh cow dung and fermentation biogas slurry	Filter paper strip, CMC	Clear zone	Zhou Hong-li et al. (2015)
B. cereus strain BR0302	Coastal Wetland Soil	CMC	Gram's iodine	Chantarasiri et al. (2015)
Bacillus sp. MSW3 *Pseudomonas* sp.SSW3 *Serratia* sp.PA1 *Pseudomonas* sp. PA2	Municipal Solid Wastes and Rice Straw Wastes	CMC	Gram's iodine	Khatiwada et al. (2016)
Meiothermus Silvanus strain UniMAP06	hot spring	CMC	Congo red and NaCl	Kunasundari Balakrishnan et al. (2016)
Stenotrophomonas rhizophila, Brevibacterium halotolerans, Achromobacter marplatensis, Bacillus methylotrophicus Pseudomonas azotoformans, Bacillus sonorensis, Bacillus subtilis, Ochrobactrum thiophenivorans	palm wastes (fiber and palm leaves), soil, woody wastes, manure, straw and sugarcane molasses	CMC	Congo red and NaCl	Saffari et al. (2016)
Bacillus subtilis (CB3) *Bacillus subtilis* (CB4) *Bacillus cereus* (CB8)	Soil and water samples	CMC	Gram's iodine	Vimal et al. (2016)
Proteus vulgaris AK1 *Serratia ficaria* AK2	Water hyacinth	CMC	Congo red and NaCl	Archana et al. (2016)
Geobacillus sp. HTA426	Hotspring	CMC	Congo red and NaCl	Potprommanee et al. (2017)

2.3.3. Cellulase activity assays

Quantitative assay to measure the cellulase activity is often done in addition to qualitative methods to validate the screening procedure. There are two basic approaches to measure cellulase activity (i) measuring the activities of individual cellulases – endoglucanase, exoglucanase and β-glucosidases, and (ii) measuring the total cellulase activity (Percival Zhang et al. 2006). These methods differ mainly in the substrates used for the assay. There are three major ways in which result of the assays can be estimated. These measure (1) the product accumulated after hydrolysis, (2) the drop in substrate quantity, and (3) the alteration in the physical properties of substrates.

Majority of the assays involve measuring the amount of hydrolysis product which includes reducing sugars, total sugars, and chromophores. Reducing sugar assay which is the most commonly used method includes Nelson-Somogyi (NS) method (Somogyi 1952), dinitrosalicyclic acid (DNS) method (Miller 1959), 4-hydroxybenzoylhydrazine (PAHBAH) method (Lever 1972), ferricyanide method (Kidby and Davidson 1973) and the 2,2′-bicinchroninate (BCA) method (Waffenschmidt and Jaenicke 1987). Total soluble sugars, can be measured directly by the anthrone-H_2SO_4 method (Roe 1955) or phenol-H_2SO_4 method (DuBois et al. 1956). Glucose can be measured by an enzymatic glucose kit. The reduction in substrate can be measured by gravimetric and chemical methods. Measurable physical cellulose properties representing cellulase activity include swollen factor, structure collapse, turbidity, fiber strength and viscosity. Both these methods are

less popular than measuring hydrolysis product (Percival Zhang et al. 2006).

The NS assay and the DNS assay are the most popular methods used by researchers for sugar estimation. Researchers like Deka et al. (2011) and Akaracharanya et al. (2014) used NS method for estimation of sugar released from CMCase assay. Fatema and Manchur (2015) quantitated all three cellulase enzyme and measured the amount of reducing sugar's released by Nelson's modification of Somogyi method. Rodrigues et al. (2010) determined the CMCase activity of recombinant *Escherichia coli* DH5α using two methods; viscosity reduction and release of reducing sugars. The reducing sugar was determined by NS method. DNS method for sugar estimation has been extensively used by researchers for measuring the sugar released by cellulase assays (Prabesh et al. 2016). Boonmee (2009) used NS method to measure reducing sugar liberated from filter paper and CMC, and DNS method for cellobiose.

2.3.4. Importance of *Bacillus* species

Cellulolytic bacteria of genus *Acetivibrio, Bacillus, Bacteroides, Cellulomonas, Clostridium, Erwinia*, *Ruminococcus,* and *Thermomonospora* have been well studied. Among them *Bacillus* spp. are the most dominant owing to the capacity of some selected species to produce and secrete high level of extracellular enzymes (Schallmey et al. 2004). The predominance of genus *Bacillus* in soil and waste with high cellulose content has been widely reported (Pourcher et al. 2001). However, the study of *Bacillus* cellulase has, until recently, lagged behind that of fungal enzymes due to the fact that they hydrolyze synthetic

carboxy methyl cellulose, but barely hydrolyze the crystalline form of cellulose (Balasubramanian and Simoes 2014). *Bacillus* strains are well-known for their spore forming ability and production of secondary metabolites like antibiotics. These strategies give them an additional advantage over competitors under conditions of slow growth on cellulosic substrates (Lynd et al. 2002). A variety of *Bacillus* species are reported to secrete cellulase, these include *B. brevis* VS-1 (Singh and Kumar 1998), *B. pumilus* BPCRI 6 (Kotchoni et al. 2003), *Bacillus amyoliquefaciens* DL-3 (Lee et al. 2008), *B. subtilis* (Kim et al. 2012), *B. cereus* MRK1 (Kumar et al. 2012b), *B. licheniformis* SM1 (Bhatt et al. 2014) and *Bacillus* sp. (Nandimath et al. 2016). *Bacillus* strains belonging to species such as *B. subtilis* and *B. sphaericus* are known to express high cellulase activities (Chan and Au 1987; Singh et al. 2004).

The ability of *Bacillus* species to produce large quantities of an array of extracellular enzymes has made them leading industrial enzyme producers. Moreover, these organisms secrete enzymes directly into the fermentation broth making the downstream processing easier (Schallmey et al. 2004). Consequently *Bacillus* species isolated from different sources are being extensively studied for production of different hydrolytic enzymes. Production of hydrolases such as amylases, proteases, and lipases from biosurfactant-producing strains of *Bacillus subtilis* has been evaluated by Barros et al. (2013). *B. thuringiensis* with a variety of enzyme capacities, such as proteases, amylases, esterases, and chitinase was isolated by Mazzucotelli et al. (2013) from agro-industrial by-products. Yadav et al. (2016) studied the production of cold active hydrolytic enzymes by psychrotrophic *Bacilli* isolated from sub-glacial

lakes of Himalayas. The enzymes analyzed were cellulase, xylanase, β-galactosidase, laccase, chitinase, amylase, pectinase, protease and lipase. Recently, Feto and Motloi (2016) extracted a variety of hydrolytic enzymes namely, cellulase, xylanase, pectinase and amylase from *B. subtilis* PFMRI and *P. macerans* PF9. As *Bacillus* spp. play important role in biotechnological applications and in industrial processes and products, the research on these genera of bacteria are still ongoing.

2.4. MATERIALS AND METHODS

2.4.1. Sample collection

Samples were collected randomly in sterile containers from different sources for the isolation of cellulolytic bacteria. The different sources screened were forest soils, compost soils, soil from municipal waste dumping ground and cowdung. The samples were stored at 4 °C until use.

2.4.2. Enrichment and isolation of cellulase producers

One gram sample was aseptically transferred to 100 ml of sterile CMC broth (Table 2.3) and incubated at 37 °C for 5 days for enrichment of cellulase producing bacteria. A loopful of the sample from the enriched culture was streaked on CMC agar plates and incubated for about 24-48 h at 37 °C. Isolated colonies were purified by re-streaking on CMC agar plates.

Table 2.3: Composition of CMC broth

Components	g/l
CMC	10
NaCl	6
$(NH_4)_2SO_4$	1.0
KH_2PO_4	0.5
K_2HPO_4	0.5
$MgSO_4$	0.1
$CaCl_2$	0.1
$NaNO_3$	0.1
Yeast extract	1.0
pH	7.0

2.4.3. Qualitative screening of cellulase producers

Cellulase production by the isolated colonies were detected by flooding the streaked plates with Congo red solution (1 mg/ml in water) for 15 min, and then de-staining with 1M NaCl solution, for 10-15 min. Enzyme production was detected by the presence of clear zone around the colonies (Teather and Wood 1982). Pure culture of selected isolates was preserved in glycerol stocks.

2.4.4. Morphological and biochemical characterization

The selected isolates were subjected to morphological and biochemical characterization. Individual colonies were morphologically characterized by studying colony characters like colour, opacity, margin and elevation. Cell morphology was studied by gram staining, endospore

staining and motility test. Further the isolates were biochemically characterized by following tests : IMViC, starch hydrolysis, catalase, nitrate reduction, oxidase, urease and carbohydrate utilization (Cappuccino and Sherman 2002). Based on the results the isolates were identified up to the genus level according to Bergey's Manual of Determinative Bacteriology (Bergey et al. 1974).

2.4.5. Quantitative screening of endoglucanase producers

The crude enzyme for quantitative assay was prepared by inoculating 1 % overnight (adjusted to a McFarland standard of 1.0, approximately $3x10^8$ CFU/ml) culture of each effective isolate in 50 ml of CMC broth and incubating at 37 °C for 24 h. Samples were taken from this production medium and centrifuged at 10,000 g for 10 min to separate the bacterial cells. These supernatants were used as the crude enzyme for quantitative assay.

Endoglucanase activity was assayed using DNS reagent (Miller, 1959) by estimation of reducing sugars released from CMC. Crude enzyme was added to 1 ml of 1 % CMC in 50 mM sodium citrate buffer (pH 5.0) and incubated at 40 °C for 30 min. After incubation, the reaction was stopped by the addition of 3 ml of DNS reagent (0.2 % phenol, 1 % DNS, 1 % sodium hydroxide and 0.05 % sodium sulfite) and boiled in water bath for 15 min. To this 1 ml Rochelle salt (40 % sodium potassium tartarate) was added (Ghose 1987). A reaction mixture with 1 ml citrate buffer instead of enzyme was kept as blank and used to set the spectrophotometer at zero absorbance. Control was maintained by incubating the substrate in similar way as test and adding DNS reagent to

stop reaction immediately after addition of enzyme. Sugars liberated were determined by measuring absorbance at 540 nm. The difference in absorbance of test and control were used to find the amount of glucose released, using glucose calibration curve. All the experiment was carried out in duplicates. The enzyme activity was calculated using the following equation

$$\text{Enzyme activity (U/ml)} = \frac{\text{µmol of glucose released x total assay volume x dilution factor}}{\text{volume of enzyme used x duration of incubation in min}}$$

2.4.6. Characterization and identification of selected isolate

2.4.6.1. *Genomic DNA extraction*

Pure genomic DNA was isolated using XcelGen Bacterial gDNA kit (Cat No: XG2411-01) following manufacturer's protocol. The protocol was as follows:

One to three ml of overnight culture was centrifuged at 12,000 g for 2 min at room temperature. The supernatant was discarded and pellet resuspended in 180 µl TE buffer or elution buffer. To this solution 18 µl of 50 mg / ml lysozyme solution and 5 µl RNase A was added and incubated at 30 °C for 15-30 min. The cells were centrifuged at 5,000 g for 5 min at room temperature and supernatant discarded leaving behind 10 µl residual liquid in the tube. The pellet was resuspended by vortexing. To this 25 mg glass beads and 200 µl Buffer TL was added. The mixture was vortexed at maximum speed for 5 min. The beads were allowed to settle down and the supernatant transferred to a new 1.5 ml centrifuge

tube. To the mixture 25 µl proteinase K was added and vortexed for 10 s. The sample was spun briefly and the mixture was incubated at 55 °C in a shaking water bath for 30 min. Around 220 µl Buffer BL was added, vortexed to mix and incubated at 65 °C for 10 min. To this 220 µl of absolute ethanol was added and mixed thoroughly by vortexing for 20 s. The entire content was transferred into a DNA mini column and centrifuged at 10,000 g for 1 min to bind DNA. The collection tube and flow-through were discarded and the column transferred into a new 2 ml tube. The column was loaded with 500 µl Buffer KB and centrifuged at 10,000 g for 1 min. The flow through was discarded and column placed in same collection tube. The column was washed by adding 650 µl DNA wash buffer (diluted with ethanol) and centrifuged at 10,000 g for 1 min. The washing step was repeated to remove contaminants. The flow-through was discarded; the column was placed back into collection tube and centrifuged at maximum speed (10,000 g) for 2 min to dry the column. The column was placed into a nuclease-free 1.5 ml microfuge tube and 50 µl of pre warmed (65 °C) elution buffer was added to DNA Mini column. It was incubated at 65 °C for 2 min and centrifuged at 10,000 g for 1 min to elute the DNA. The elution step was repeated with 50 µl elution buffer.

2.4.6.2. *Analysis of DNA*

The DNA having 260:280 ratio between 1.8 – 2.0 was considered of good quality and was used for further analysis. The quality of DNA was also assessed using agarose gel electrophoresis. 1 % agarose gel was prepared in 1X TBE buffer. The gel was melted in microwave, cooled and 5 µl of ethidium bromide (EtBr) was added. The comb was placed on

casting tray; gel was poured and allowed to solidify. The comb was removed and the casting tray was kept in the electrophoretic unit. The electrophoretic chamber was filled with 1X TBE buffer until it covers the top of the gel. The samples were loaded with 6X bromophenol blue loading dye. A 100 bp DNA ladder was loaded and the gel was run for 30 min at 80 V. The gel was viewed using gel documentation system. This DNA sample was used as template for amplification of 16S rRNA gene.

2.4.6.3. *PCR amplification of 16S rRNA gene*

The 16S rRNA gene was amplified from genomic DNA by polymerase chain reaction using universal primers

8F: 5'-AGAGTTTGATCCTGGCTCAG-3' and

1492R: 5′-ACG GCTACCTTGTTACGACTT-3′

The PCR reaction was carried out in 25 µl final volume (Table 2.4).

Table 2.4: Composition of PCR reaction mixture

Components	Volume (µl)
Nuclease free water	7.5
2X PCR master mix (MBI Fermentas)	12.5
Forward primer 8F	1.0
Reverse primer 1492R	1.0
Diluted DNA (30 ng/µl)	3.0

The protocol for PCR was as follows

Table 2.5: PCR amplification profile

Initial denaturation	Denaturation	Annealing	Extension	Final Extension
95 °C	94 °C	52 °C	72 °C	72 °C
2 min	30 s	30 s	90 s	10 min
	30 cycles			

The amplified product was run on 1 % agarose gel at 80 V for 30 min and visualized using gel documentation system. A 100 bp DNA ladder was used as the standard for molecular weight determination.

2.4.6.4. ***Elution of amplified product***

XcelGen DNA Gel/PCR Purification Mini Kit (Cat No: XG3511-01/3514) was used for purification of amplified product after excision from gel .The protocol was as follows:

Firstly the spin column was prepared for proper binding of DNA. For this 400 µl of Buffer BL was added into the spin column, incubated at room temperature for 2 min, then centrifuged for 2 min at 12,000 rpm and discarded the flow-through. The DNA fragment was excised from the agarose gel and weighed in a 1.5 ml microfuge tube. Three volumes of buffer GC was added to one volume of gel in the micro centrifuge tube and incubated at 55-60 °C for 8 min. The tube was mixed by tapping the bottom every 2 min till the gel has melted completely. The tube was cooled to room temperature and 1 volume of isopropanol was added and mixed well. The spin column was placed in collection tube and upto

700 µl DNA/Buffer GC mixture was transferred into the column. The column was centrifuged at 13,000 g for 1 min at room temperature and the flow through was discarded. This step was repeated to process the remaining solution. To the column 500 µl of Buffer GC was added and centrifuged at 13,000 g for 1 min at room temperature. The column was washed twice by adding 650 µl DNA Wash Buffer and centrifuging at 13,000 g for 1 min at room temperature. The flow through was discarded and the empty column was centrifuged at 13,000 g for 2 min to dry the ethanol residue in the matrix. The column was transferred into a clean 1.5 ml microfuge tube and 30-50 µl pre-warmed (60 °C). Elution Buffer was added to the center of the column. It was incubated at room temperature for 1 min and centrifuged at 13,000 g for 1 min to elute the DNA. The eluted DNA solution was reloaded into the column for second elution.

2.4.6.5. *Sequencing and analysis of PCR product*

The amplicon was sequenced from Xcelris Labs Ltd., Ahmedabad, India using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) on ABI 3730xl Genetic Analyzer (Applied Biosystems). The sequence was edited manually to correct falsely identified bases and trimmed to remove unreadable sequence at the 3' and 5'ends (considering peak and Quality Values for each base) using the sequence analysis tools. The edited sequence was then used for similarity search with the nr database using BLAST program of NCBI genbank for identifying the bacterial strain.

2.4.6.6. *Construction of phylogenetic tree*

The consensus sequence generated from forward and reverse sequence data using aligner software was used to carry out BLAST with the nr database of NCBI genbank. Based on maximum identity score, best twenty sequences with 99 % to 100 % similarity were selected and a phylogenetic tree was constructed with the candidate strain. Multiple sequence alignment was performed using ClustalW and evolutionary history inferred by the neighbor-joining method. Kimura 2-parameter method was used to compute the evolutionary distances and MEGA 5 (Tamura et al. 2011) was used for phylogenetic analysis. The confidence of tree topologies was estimated using bootstrap resampling analysis for 1000 replicates and the tree generated was visualized using FigTree v1.4.2.

2.4.6.7. *Scanning Electron Microscopy (SEM)*

Overnight culture of *Bacillus subtilis* MU S1 was centrifuged at 8000 rpm for 6 min to pellet the cells. The pellet was resuspended in fixative (5 % gluteraldehyde in 0.1 M phosphate buffer-pH 7.2) and incubated for 30 min at room temperature. After incubation, the culture was pelleted and washed twice in 0.1 M phosphate buffer. The pellet was resuspended in 1 % osmium tetroxide (prepared in 0.1 M phosphate buffer-pH 7.2) and incubated for 1 h at room temperature. The pellet obtained after centrifugation was washed twice with sterile distilled water. The dehydration of pellet was carried out in ethanol series of 35 % to

100 % and kept in desiccator overnight. The sample was mounted on specimen stubs and viewed under SEM.

2.4.7. Effect of physical parameters on growth

The growth condition of the identified isolate was optimized by inoculating it in CMC broth and incubating at different temperatures (30 °C, 40 °C and 50 °C) under static and shaker condition (140 rpm). The culture was withdrawn after 24 h and analyzed for bacterial growth by measuring the absorbance at 600 nm.

2.4.8. Time course profile of cellulolytic enzyme production

The crude enzyme for quantitative assay was prepared by inoculating 50 ml of CMC broth with 1 % overnight (adjusted to a McFarland standard of 1.0, approximately $3x10^8$ CFU/ml) culture and incubating at 40 °C for 48 h in an orbital shaker (140 rpm). CMC was used as carbon source in the media for cellulase production. The samples were collected at every 12 h interval and centrifuged at 10,000 g for 10 min to separate the bacterial cells. The supernatants were used as crude enzyme for quantitative assay.

Endoglucanase (CMCase) assay was performed by incubating 1 ml of crude enzyme with equal volume of 1 % CMC substrate in 50 mM sodium citrate buffer (pH 5.0) for 30 min at 40 °C. Exoglucanase (avicelase) and β-glucosidase (cellobiase) activity assays were conducted similarly with 1 % avicel and cellobiose as substrate respectively. Filter paper activity (FPase) assay for total cellulases was carried out using 50 mg strip of Whattman filter paper No. 1 (1 x 6 cm) under the same

conditions with incubation time of 1 h (Ghose 1987). The amount of sugar released was measured by DNS method (Miller 1959). Control was maintained as mentioned earlier. All experiments were carried out in duplicates and enzyme activity was calculated using equation given section 2.4.5. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1µmol of glucose per minute under the assay conditions.

2.4.9. Multifarious enzyme production potential of *Bacillus subtilis* MU S1

The ability of the selected strain for production of diverse enzymes was evaluated by studying the production of different biomolecules. The production of different enzymes was measured using agar well diffusion method on nutrient agar plate supplemented with 1 % of corresponding substrates. The substrates used were casein, pectin, starch, birch wood xylan and tannic acid for the enzymes protease, pectinase, amylase, xylanase and tannase respectively. Wells of 5 mm diameter were cut under sterile conditions into the agar plates and 50 µl of overnight grown cell supernatant was added. The plates were incubated at 40 °C for 24-48 h. After incubation the enzyme activities were measured by flooding the plate with appropriate dyes. In case of amylase and pectinase, iodine solution (0.25 % iodine, 0.5 % potassium iodide and 31 ml of 20 % ethanol) was added to visualize the zone (Cappuccino and Sherman 2002). Xylanase activity was visualized by staining with Congo red dye (0.1 %) for 15 min and destaining with 1M NaCl for 10 min

(Meddeb-Mouelhi et al. 2014). Protease (Choudhary and Jain 2012) and tannase (Kumar et al. 2010) activities were observed as clear and brown zone respectively, around the well after incubation. The diameter of the zone around the well was measured to quantify the enzyme activities.

The ligninolytic potential of the strain was studied by monitoring the decolourization of synthetic lignin-like dyes (malachite green and methylene blue). Mineral salt medium (Table 2.6 and 2.7) supplemented with 0.1 % glucose and yeast extract was used. Dyes were added in the concentration of 50 mg/l (malachite green) and 25 mg/l (methylene blue) (Bandounas et al. 2011). Wells were cut and 50 µl of cell free supernatant was added. The plates were incubated for 48 h at 40 °C. The dye degrading ability was detected by clear zone around the plates. The diameter of the zone around the well was measured to quantify the ligninolytic ability.

Table 2.6: Composition of MSM agar

Components	**g/l**
Na_2HPO_4	3.6
$(NH_4)_2SO_4$	1.0
KH_2PO_4	1.0
$MgSO_4$	1.0
$CaCl_2$	0.1
Trace element solution	10 ml
Agar	15
pH	7.0

Table 2.7: Composition of trace element solution

Components	g/l
$ZnSO_4$	0.001
$MnCl_2$	0.003
$NiCl_2$	0.002
Na_2MoO_4	0.003
H_3BO_3	0.03
$CuCl_2$	0.001

2.5. RESULTS

2.5.1. Screening and isolation of cellulolytic bacteria

Among the different samples collected, forest soil samples were found to be rich in cellulose degraders. Thirty two isolates showing visible zone of clearance were isolated among which 24 isolates were obtained from forest soil (collected from different forests in Munnar wildlife division), 4 isolates were obtained from compost soil and two each from municipal dumping ground and cowdung (Table 2.8). Among the isolated bacteria four with largest zone of clearance (Figure 2.1) were selected based on zone diameter. The zone diameters obtained were MU S1 (23 mm), MU S2 (6 mm), MU S3 (19 mm) and MU S4 (15 mm).

Table 2.8: Zone diameter of isolated strains

Isolate code	Zone diameter (mm)
Munnar wildlife division	
MU S1	23
MU S2	6
MU S3	19
MU S4	15
MU S5	4
MU S6	2
MU S7	NM
MU S8	3
MU S9	NM
MU S10	5
MU S11	3
MU S12	2
MU S13	5
MU S14	NM
MU S15	3
MU S16	NM
MU S17	NM
MU S18	5
MU S19	NM
MU S20	NM
MU S21	4
MU S22	5
MU S23	2
MU S24	NM
Compost soil	
CS S1	3
CS S2	4
CS S3	4
CS S4	NM
Dumping grounds	
DG S1	5
DG S2	2
Cow dung	
CD S1	4
CD S2	NM

NM: Zone very diffuse and not measurable

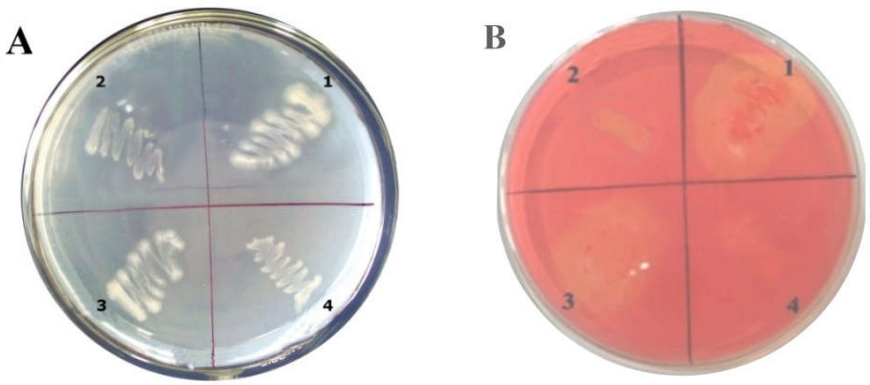


Figure 2.1: Isolation of cellulolytic bacteria **(A)** selected bacteria on CMC plate **(B)** Congo red plate assay of the selected isolates

2.5.2. Morphological and biochemical characterization

The isolates were characterized using morphological and biochemical methods. The colony characteristics were observed and biochemical tests were performed. The results were tabulated (Table 2.9). Based on the results the isolates were identified upto genus level according to Bergey's Manual of Determinative Bacteriology. Isolate MU S1 and MU S3 belonged to *Bacillus* sp., whereas MU S2 and MU S4 were members of the genus *Staphylococcus* and *Corynebacterium* respectively.

Table 2.9: Morphological and biochemical characteristics of selected organisms

Characteristics	Isolate code			
	MU S1	**MU S2**	**MU S3**	**MU S4**
Colony				
	Irregular, flat, lobate, opaque, cream	Circular, convex, entire, translucent, cream	Irregular, flat, lobate, opaque cream	Circular, flat, entire, opaque, cream
Morphological				
Shape	Rods	Cocci	Rods	Rods
Gram staining	+	+	+	+
Motility	+	-	+	-
Spore formation	+	-	+	-
Physiological				
Indole	-	-	-	-
Methyl-red	-	-	+	+
Voges-Proskauer	+	-	-	-
Citrate	+	+	-	-
Nitrate	+	-	+	+
Urease	-	-	-	-
Catalase	+	+	+	+
Oxidase	+	+	+	+
Sugar utilization				
Glucose	+	+	+	+
Lactose	-	+	-	-
Sucrose	+	+	+	-
Mannitol	+	+	+	-

2.5.3. Quantitative screening of endoglucanase producers

The endoglucanase activity was determined after 24 h incubation at 37 °C. The strains belonging to *Bacillus* spp. showed comparatively higher endoglucanase activity. *Bacillus* sp. MU S1 exhibited activity of

178.54 U/ml and *Bacillus* sp. MU S3 showed an activity of 98.43 U/ml. This was followed by *Corynebacterium* sp. MU S4 and *Staphylococcus* sp. MU S2 with 76.91 and 24.84 U/ml respectively (Figure 2.2).

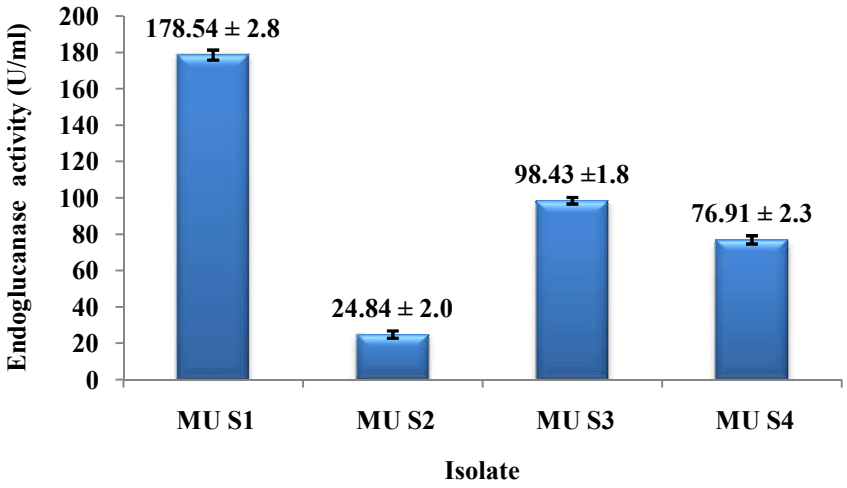


Figure 2.2: Comparison of endoglucanase activities of selected isolates

2.5.4. Characterization and identification of strain *Bacillus* sp. MU S1

For species level identification of strain MU S1, molecular and phylogenetic analyses were carried out. Genomic DNA was extracted and PCR was done using universal primers (8F and 1492R). The PCR product was run on agarose gel and a single band of 1500 bp was obtained (Figure 2.3). The band was eluted from the gel and sequenced. The consensus sequence of 1362 bp generated from forward and reverse sequence data was used for BLAST analysis. The BLAST results revealed 99 % similarity with *Bacillus subtilis* strain BCX-1, *Bacillus licheniformis* strain TAD17, *Bacillus* sp. KT132, *Bacillus* sp. KT52, *Bacillus* sp. KT107, *Bacillus* sp. KT103, *Bacillus* sp. KT113, *Bacillus tequilensis* strain SML11, *Bacillus* sp. SE-54, *Bacillus* sp. JBP-21, *Bacillus* sp. KT10,

Bacillus sp. BAB-4136, *Bacillus* sp. BAB-4163, *Bacillus* sp. S22217, *Bacillus* sp. CA-2, *Bacillus subtilis* strain BF8, *Bacillus subtilis* strain LD181, *Bacillus amyloliquefaciens* strain BF10, *Geobacillus stearothermophilus* strain BK17-2366 and *Bacillus subtilis* strain GY-57.

A phenogram reflecting the relationship among the strain and candidate sequence of related strains obtained from NCBI database are presented in Figure 2.4. The phylogenetic analysis displayed close similarity with *Bacillus* sp. JBP-21 (KM675950.1) and *Bacillus subtilis* strain LD181 (KJ564129.1). From results of biochemical and molecular analysis the isolate was affirmed to belong to *B. subtilis*. The sequence was deposited in Genbank database with accession number KT715518.

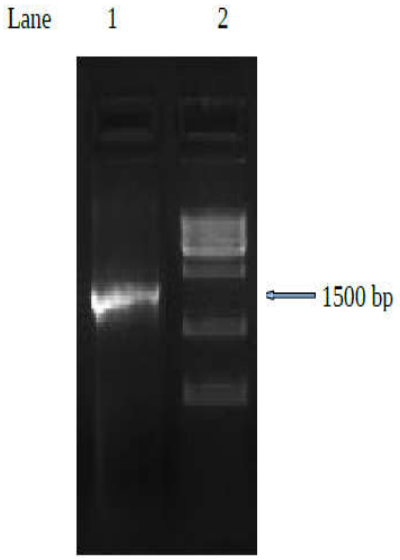


Figure 2.3: PCR amplification product run on agarose gel. Lane 1:16S rDNA amplicon band, lane 2: DNA ladder

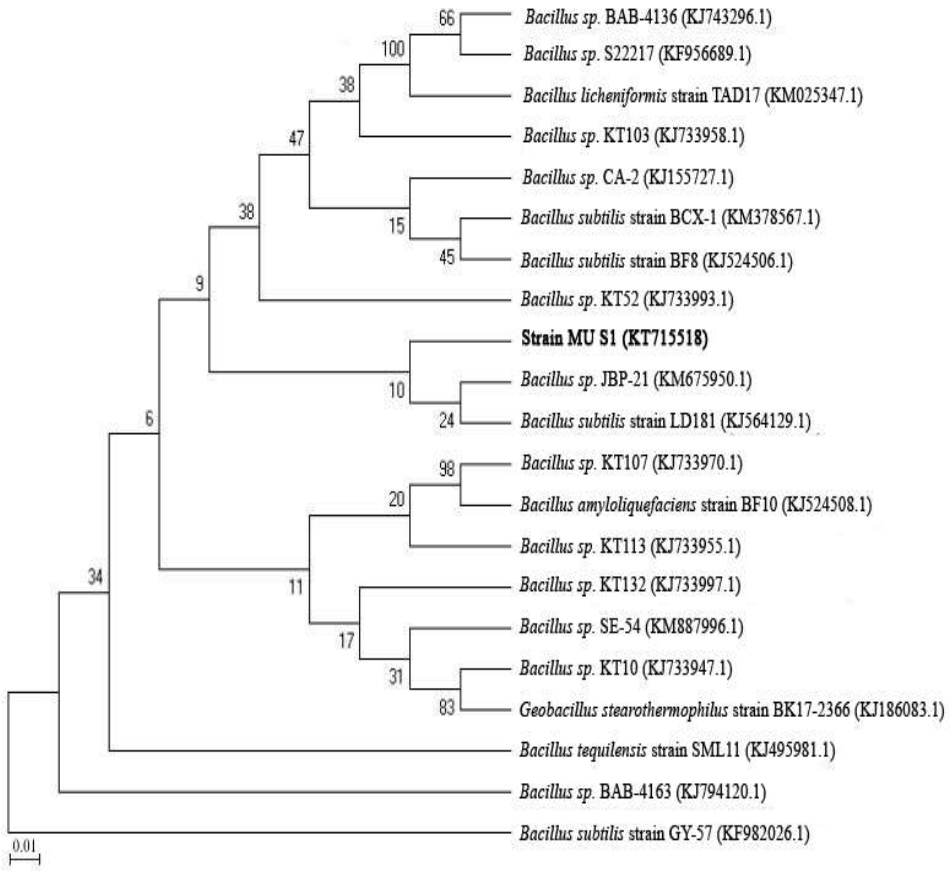


Figure 2.4: Phylogenetic tree based on 16S rRNA gene sequence, showing relationship between the isolate MU S1 and closely related strains

SEM analysis result showed rods of width and length of approximately 0.4–0.5 µm and 1.4–1.8 µm respectively (Figure 2.5).

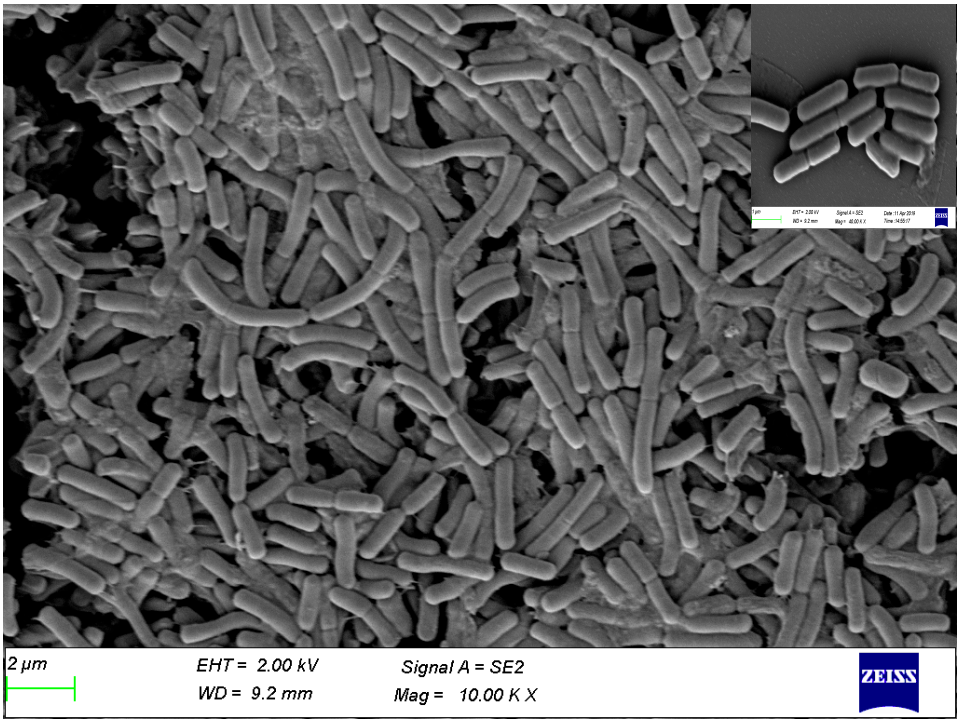


Figure 2.5: SEM image of *B. subtilis* MU S1

2.5.5. Optimization of growth conditions

To find the optimum growth conditions of *Bacillus subtilis* MU S1, it was grown at different temperatures under static and shaking (140 rpm) conditions (Figure 2.6). Even though the cultures showed growth at 30 °C, 40 °C and 50 °C the optimum growth was at 40 °C under agitated condition.

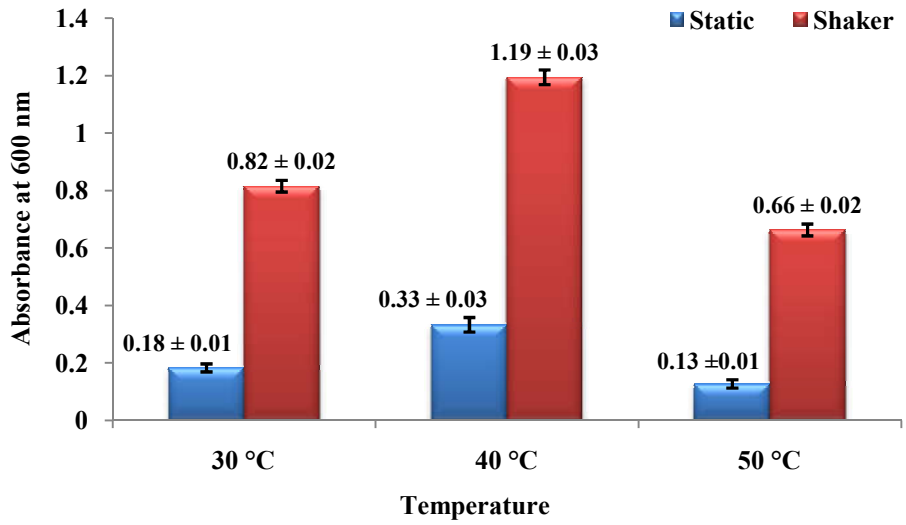


Figure 2.6: Growth profile of *B. subtilis* strain MU S1 under different conditions after 24 h incubation

2.5.6. Time course profile of cellulolytic enzyme production

The cellulolytic potential of the strain was examined after inoculating in production medium and incubating at 40 °C in an orbital shaker. The activity was assayed at 12 h intervals and the results were recorded. It was observed that strain MU S1 very effectively utilized CMC for growth by secreting multiple enzymes (endoglucanase, exoglucanase, β-glucosidase and FPase) extracellularly. The results were tabulated (Table 2.10) and also represented graphically (Figure 2.7).

Table 2.10: Cellulase activities of *Bacillus subtilis* MU S1 during 48 h incubation

Enzyme	12 h	24 h	36 h	48 h
Endoglucanase (U/ml)	129.53 ± 3.3	**185.76 ± 4.6**	161.12 ± 3.5	133.95 ± 3.3
Exoglucanase (U/ml)	74.56 ± 4.0	**151.64 ± 5.9**	118.78 ± 5.6	93.51 ± 4.9
β –glucosidase (U/ml)	78.35 ± 2.7	113.09 ± 4.6	152.9 ± 6.5	**156.02 ± 3.9**
FPase (U/ml)	50.86 ± 3.6	**58.44 ± 3.1**	45.95 ± 2.3	42.02 ± 1.4

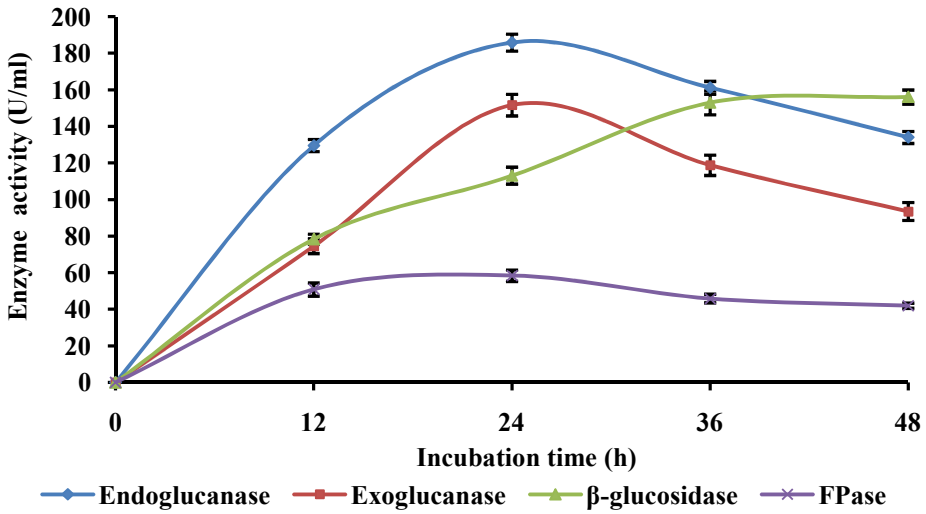


Figure 2.7: Cellulase activity profile of *Bacillus subtilis* MU S1

2.5.7. Multifarious enzyme production potential of *Bacillus subtilis* MU S1

The ability of the strain MU S1 to produce different hydrolytic enzymes and to degrade lignin like dyes was evaluated using agar well diffusion method. The strain effectively utilized the different substrates as

indicated by a zone around the well (Figure 2.8). The zone diameter obtained were as follows : amylase (16 mm), pectinase (19 mm), xylanase (18 mm), protease (24 mm), tannase (16 mm), malachite green (15 mm) and methylene blue (18 mm).

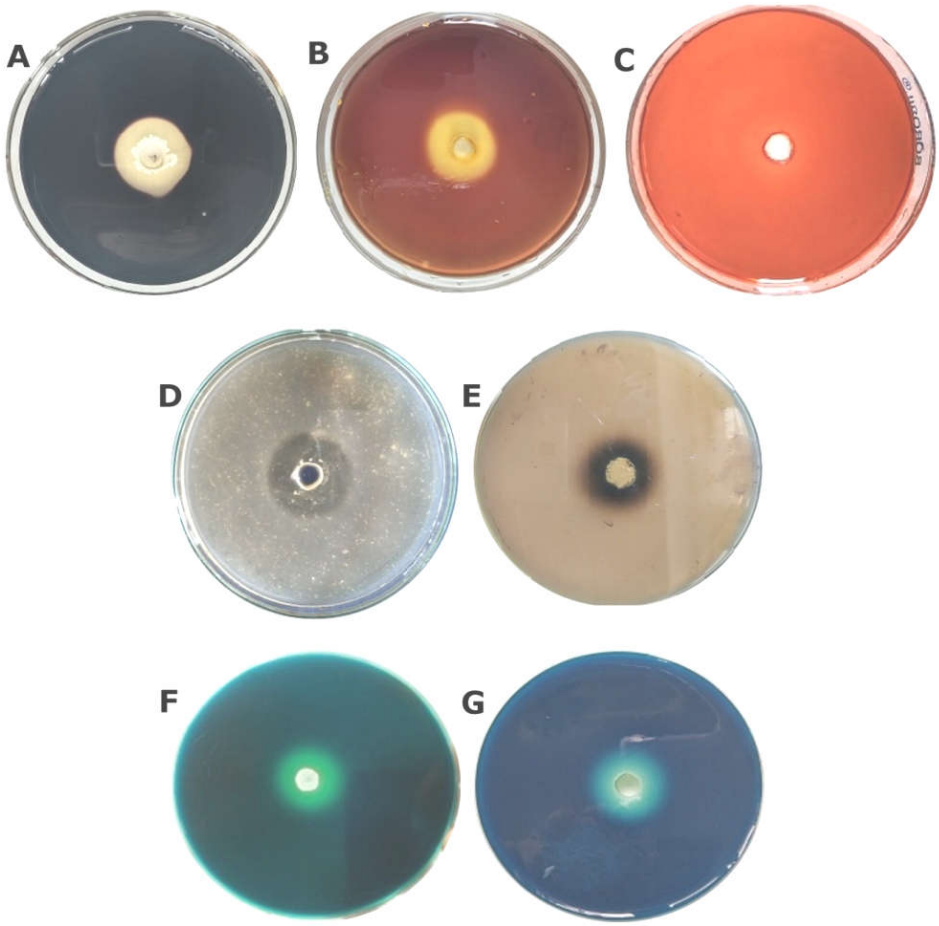


Figure 2.8: Screening for multifarious activities of *B. subtilis* MU S1 by agar well diffusion assay **(A)** amylase **(B)** pectinase **(C)** xylanase **(D)** protease **(E)** tannase **(F)** malachite green **(G)** methylene blue

2.6. DISCUSSION

Cellulases have attracted continuing interest of biotechnologists due to their applications in diverse industries, especially in cellulosic biofuel production. Thus recently major research has aimed at isolation and identification of novel and highly active cellulase producers. Soil is one of the best sources of extracellular enzyme producers.

Samples were collected from different sources and screened for cellulase producing bacteria, after enrichment in CMC broth. Thirty two colonies displayed visible zone of clearance after staining with Congo red. Among this majority of isolates (24) were obtained from soil samples collected from different forests under Munnar wildlife division. The forests in Munnar were preferred because of the abundance of unexplored microbial diversity. Our study established that the soil from this area is rich reservoir of cellulolytic bacteria. This was in accordance with results of Hatami (2008).

Among the positive isolates, four with zone diameter ranging from 6 – 23 mm were selected and biochemically characterized. Two strains belonged to *Bacillus* sp. (MU S1 and MU S3) and one each to *Staphylococcus* sp. (MU S2) and *Corynebacterium* sp. (MU S4). On quantitative analysis it was observed that *Bacillus* sp. MU S1 exhibited highest endoglucanase activity of 178.54 ± 2.8 U/ml at 37 °C after 24 h incubation. This strain was isolated from soil sample collected from Eravikulam National Park which is a part of Munnar wildlife division. Several works on isolation of cellulolytic bacteria have shown that *Bacillus* spp. often surpass other genera in the amount of cellulase

produced. Fatema et al. (2015, 2016) in two of their works screened different forest soils for cellulolytic bacteria and found the *Bacillus* spp. were promising cellulase degraders among various other isolates (Fatema and Manchur 2015; Fatema et al. 2016). Researcher like Kumar et al. (2012b) and Lugani et al. (2015) also found *Bacillus* spp. to be best cellulase producers among the other isolates.

On morphological, biochemical and molecular characterization the isolate was identified as *Bacillus subtilis*. The 16S rRNA gene sequence was deposited in Genbank database with accession number KT715518. *Bacillus subtilis* strains with cellulolytic potentials have been isolated from variety of sources like water and soil of the Amazon region (Heck et al. 2002), agricultural soil (Kim et al. 2012), termites (Tarayre et al. 2014), fecal samples of Tibetan pigs (Meng et al. 2014) and snail (Dar et al. 2015).

The growth parameters of *Bacillus subtilis* MU S1 were optimized by growing the bacterium at different temperatures and under agitated (140 rpm) and non agitated conditions. It was observed that agitation at 40 °C was the optimum condition for growth. This was in accordance with Sethi et al. (2013) who recorded an optimum growth at 40 °C for *Bacillus* sp. Jansová et al. (1993) reported maximum specific growth rate of *Bacillus subtilis* 115 in the temperature range of 45–48 °C. In agreement with our observation, a positive effect of agitation on growth and enzyme production by *Bacillus subtilis* was reported by Deka et al. (2013).

The ability of the strain to produce different celluloytic enzymes (endoglucanase, exoglucanase and β–glucosidase) was studied by growing it in CMC media and performing the activity assay using the respective substrates. Cellulolytic activity profile of *Bacillus subtilis* MU S1 was obtained during incubation in CMC broth for 48 h. Endoglucanase and exoglucanase activity reached a peak at 24 h and decreased thereafter. β-glucosidase activity which was an exception to this case was found to rise even after 24 h incubation. Similar incubation time was reported in *Bacillus subtilis* AS3 (Deka et al. 2011). Whereas Fatema et al. (2016) recorded varied incubation times for optimum endoglucanase production by different *Bacillus* spp. They observed maximum endoglucanase production by *Bacillus sphaericus* (GK1) and *Bacillus pumilus* (JK1) after 4 and 5 days respectively. The main reason for decrease of endoglucanase and exoglucanase activity after 24 h is the catabolite repression of cellulase gene by glucose, which is the major end product of cellulose digestion (Chan and Au 1987). Another reason for the decrease could be due to feedback inhibition of endoglucanases and exoglucanases by cellobiose. On further incubation as cellobiose accumulates β- glucosidase production increases. However, at the end of 48 h the activity tends to remain constant which may be due to feedback inhibition of β-glucosidase by glucose.

Bacillus subtilis MU S1 exhibited higher endoglucanase activity (185.76 ± 4.6 U/ml) compared to other cellulolytic enzymes. This is in agreement with previous studies which also recorded a CMCase activity greater than exoglucanase, β- glucosidase and FPase (Pason et al. 2006; Saratale et al. 2012). The endoglucanase activity obtained in the present

study was higher than that exhibited by some known natural isolates, for example, *Geobacillus* sp. HTA426 isolated from hot spring (54.38 ± 0.01 U/ml) (Potprommanee et al. 2017). Whereas, similar activities were obtained from *Stenotrophomonas* sp., MY6 (137.36 U/ml) and *Bacillus cereus* FY2 (177.58 U/ml) isolated from fresh cowdung and fermentation biogas slurry (Zhou Hong-li et al. 2015).

Exoglucanase activity measured using avicel as substrate was found to reach maximum (151.64 ± 5.9 U/ml) at 24 h incubation. Comparable avicelase activities were obtained from *Bacillus sphaericus* (GK1) and *Bacillus pumilus* (JK1) isolated from soil under *Dipterocarpus* and *Lagerstroemia* forest (Fatema et al. 2016) and from *Bacillus brevis* AK5 isolated from Acacia forest soil (Fatema and Manchur 2015). Generally, avicelase activity is found to be lower than CMCase and β-glucosidase (Waghmare et al. 2014; Kumar and Parikh 2015). In a study carried out by Soares et al. (2012) 46.9 % of the isolates displayed the presence of endoglucolytic activity, 9.1 % showed exoglucolytic activity, while only a minority (4.72 %) could degrade both the substrates on plate assay.

β-glucosidase (cellobiase) activity of the strain increased beyond 24 h and then remained almost constant upto 48 h. The highest activity obtained was 156.02 ± 3.9 U/ml. Lower cellobiase activities were observed in different *Bacillus* spp. (Fatema and Manchur 2015; Fatema et al. 2016). Our results show some contrast to earlier findings of Kim et al. (2012) in which *Bacillus subtilis* strains were unable to secrete cellobiase, although its presence was detected in cell debris indicating its membrane association. Kim et al. (2004) also reported lack of extracellular

β-glucosidase in *Bacillus licheniformis* NLRI X-33. FPase (total cellulase) assay performed using filter paper as substrate, measures the ability of crude enzyme to act on both amorphous and crystalline cellulose. Since degradation of filter paper requires the concerted action of endo and exo cellulases, this assay is the best measure of total cellulase activity. The isolate MU S1 displayed highest FPase activity of 58.44 ± 3.1 U/ml at 24 h incubation.

In order to assess the industrial applicability of the strain MU S1 it was tested for the production of an array of hydrolytic enzymes. It was observed that the strain degraded all the substrates, by production of the respective enzymes (amylase, protease, pectinase, xylanase and tannase), but at different degrees. Plate method is the best method for rapid detection of specific extracellular enzymes. Researchers namely Barros et al. (2013), Mazzucotelli et al. (2013), Feto and Motloi (2016) and Yadav et al. (2016) have evaluated the hydrolytic enzyme production by different strains of *Bacillus*.

Decolorization of dye is often associated with the process of extracellular oxidases, particularly manganese peroxidases. All the enzymes involved in lignin degradation such as liginin peroxidase (Lip), manganese-dependant peroxidase (MnP) and laccase, have been reported to decolourize dyes (Singh and Singh 2010). Thus the ligninolytic potential of the strain was evaluated by establishing its ability to decolourize synthetic, lignin-like dyes. *B. subtilis* strain MU S1 decolorized the dyes as evidenced by clear zone around the well. Similar study conducted by Bandounas et al. (2011) showed that among the three isolates obtained from forest soils, strain belonging to *Bacillus* sp. (strain

LD003) showed extensive dye-decolourizing capacity, particularly towards methylene blue, Azure B and Toluidene Blue O. The potential of *B. subtilis* MU S1 to produce multiple enzymes makes it a prospective candidate for a variety of industrial applications mainly biofuel industry.

2.7. CONCLUSION

The ever-increasing demand for highly competent enzymes has accelerated the search for novel enzyme producers from unexplored environments. This study provides evidence for the existence of potent microbes of industrial importance in protected forests of Munnar wildlife division. A novel strain of *Bacillus subtilis* (strain MU S1), which produced promising levels of cellulases (endoglucanase, exoglucanase, β-glucosidase, FPase) was successfully isolated from Eravikulam National Park of Munnar wildlife division. The strain could produce different enzymes like xylanase, pectinase, protease, tannase, amylase and ligninase. The ability of the isolate MU S1 to produce multiple enzymes makes it a promising nominee for bioconversions and other industrial applications.

3.1. INTRODUCTION

Endo-β-1,4-glucanase or CMCase [EC 3. 2. 1.4] is the primary enzyme of the multienzyme complex –cellulases. It hydrolyses the cellulose chains internally providing new chain ends for other cellulases to act synergistically. Endoglucanase has potential application in many industries like detergent, biofuel, textile, paper recycling, juice extraction and animal feed additives (Bhat 2000). Consequently, there has been growing demand for endoglucanase, especially those with high activity and stability under extreme pH and temperature.

The cost of enzymes is one major factor determining the economics of a biocatalytic reaction. Various factors like temperature, pH, aeration and media constituents are known to influence enzyme production. Both physical and nutritional parameters play a crucial role in enhancing cell growth and thereby accumulation of product (Ibrahim and Elkhidir 2011). Medium constituents contribute about 30-40 % of the production cost of industrial enzymes and thus a considerable reduction in cost can be achieved by optimizing the media constituents. Accordingly, optimization studies (physical and nutritional parameters) are inevitable and forms an essential part of all enzyme research.

Conventionally, one-factor-at-a-time/one-variable-at-a-time (OFAT/ OVAT) approach, which involves changing one factor at a time while keeping the others at constant levels, has been used for media optimization studies. This technique has major disadvantages: it does not take into account the complex interactions among various components, moreover, it is time-consuming and requires a number of experiments to determine the

optimum levels (Zambare and Christopher 2011). These shortcomings can be overcome by using Design of Experiments (DOE) method. This statistical technique allows screening and selection of the factors that have a great influence on the output, followed by optimization of their levels. Full factorial design, fractional factorial design and response surface methodology are the statistical tools commonly used.

The full factorial design gives full information but is laborious as it involves a huge number of experiments making this method impracticable when large numbers of factors are being considered. The Plackett-Burman design is a two-level fractional factorial design, which facilitates the screening and selection of essential parameters and also gives convincing information about all the parameters. As compared to earlier design it is rapid, effective and time-saving (Plackett and Burman 1946). Response Surface Methodology (RSM) is a statistical and mathematical tool used for optimization of selected factors (Myers et al. 2016). This technique helps in finding the optimal levels of each variable, their interaction with each other and the net effect on the output (Ali et al. 2013). Recently, Plackett-Burman Design and RSM have been successfully used to optimize many fermentation media.

3.2. OBJECTIVES

The aim of the present chapter was to identify the optimal levels of critical parameters for enhanced production of endoglucanase by *Bacillus subtilis* MU S1. Physical parameters and medium constituents were optimized using OFAT and statistical techniques respectively.

3.3. REVIEW OF LITERATURE

Production cost is believed to be the major bottleneck in many biotechnology-based industrial processes. Fermentation media contributes a lot to the production cost and thus formulation of a suitable media is of critical importance. The fermentation media affects not only the product yield but also volumetric productivity (Singhal and Swaroop 2013). Cellulase production is influenced by a number of factors like culture conditions – including media components and physical parameters, nature of substrate and also potential of the strain used. However, the ultimate production of the enzyme depends on the interrelationship between these factors (Lynd et al. 2002). Thus, for designing a suitable production media, the fermentation conditions (temperature, pH, agitation speed etc.) and media constituents (carbon, nitrogen, metal ions etc.) must be carefully identified and optimized. Although bacteria are more tolerant to extreme environmental conditions compared to other microorganisms, each species has its own characteristic range of values at which it shows maximum growth and metabolic activity.

3.3.1. Factors affecting endoglucanase production

3.3.1.1. Fermentation conditions

The physicochemical parameters that generally affect extracellular enzyme production are temperature, pH and dissolved oxygen.

3.3.1.1.A. *Temperature*

Incubation temperature is one major factor that affects a variety of cell metabolic processes such as denaturation of protein, enzyme activity

and growth of microbes. According to Jung et al. (1974) temperature affects the specific growth rate and also physiological properties of the strain. They found that the secretion of enzymes is greatly dependent on the growth temperature. The optimization of temperature is thus a crucial factor for higher production of the enzyme.

The optimum incubation temperature for endoglucanase production varies depending on the microorganisms. Sethi et al. (2013) reported 40 °C as optimum temperature for endoglucanase production by *Pseudomonas fluorescens, Bacillus subtilis, E. coli,* and *Serratia marcescens.* The same optimum temperature was recorded for endoglucanase from *Cellulomonas, Bacillus,* and *Micrococcus* sp. by Immanuel et al. (2006). Optimum endoglucanase production at 37 °C was observed for *Paenibacillus curdlanolyticus* B-6 (Waeonukul et al. 2009) and *Paenibacillus polymyxa* (Kumar et al. 2012a). Whereas,Liang et al. (2014) obtained maximum endoglucanase activity after incubation at a temperature below 30 °C by a strain of *P. terrae*. Acharya and Chaudhary (2012) found a temperature of 50 °C as optimum for endoglucanase production by *Bacillus licheniformis* MVS1 and *Bacillus* sp. MVS3.

3.3.1.1.B. *pH*

Bacteria are very sensitive to the hydrogen ion concentration of their environment. The pH of growth medium plays a crucial role in enzyme secretion. The pH change during the growth of microbes affects the stability of the enzyme in the media. Variation in pH may bring about an alteration in charge and shape of the protein, leading to loss of catalytic property.

Cellulase production has been detected over a range of pH. Acidic pH for optimum enzyme production is usually found in fungi whereas bacteria prefer near neutral to slightly alkaline pH for enhanced enzyme production. Initial pH near neutrality was found to be most favorable for *P. curdlanolyticus* B-6 (Waeonukul et al. 2009), *P. polymyxa* (Kumar et al. 2012a), *Bacillus amyloliquefaciens* SS35 (Singh et al. 2014b), *Bacillus subtilis* AS3 (Deka et al. 2013), *Bacillus* sp. (MTCC10046) (Sadhu et al. 2014). While researchers like Kazemi et al. (2014), Liang et al. (2014), Thakkar and Saraf (2014), and Dave et al. (2015) noted high endoglucanase production when the initial pH of the medium was slightly alkaline.

3.3.1.1.C. *Agitation rate*

Agitation rate is an important factor that determines the level of dissolved oxygen in the medium and affects both growth and enzyme production by microorganisms (Jo et al. 2008). The requirement of oxygen varies depending on the microbe. Oxygen is the terminal electron acceptor for oxidative reactions and provides energy for cellular activities. Proper agitation is a must to achieve proper mixing, and mass and heat transfer (Amadi and Okolo 2013). There is a critical concentration of dissolved oxygen below which changes can occur in metabolism pathway. According to Feng et al. (2003) high agitation speed increases the dissolved oxygen and dispersion of macromolecules in the medium and thus contributing to high cell growth and enzyme production. But higher speed causes shearing effect and leads to cell death and enzyme inactivation.

From the literature review, it was clear that agitated condition was preferred over static for cellulase production by most of the bacteria. A study conducted by Hussain et al. (2017) showed that the bacteria *B. megaterium* BMS4, *B. subtilis* BTN7A, and *B. amyloliquefaciens* SA5 produced higher endoglucanase after incubation under shaking condition whereas *Anoxybacillus flavithermus* BTN7B showed higher enzyme activity under static condition. The agitation speed of 120 rpm was found to be favourable for endoglucanase production by *Bacillus subtilis* AS3 (Deka et al. 2013), *Lactococcus lactis* and *Cellulomonas fimi* (Shinde and Sharma 2014), *Bacillus* sp. Y3 (Lugani et al. 2015), and *Bacillus subtilis* K-18 (Irfan et al. 2017). Slightly higher agitation speed (150 rpm) was preferred by *Brucella* sp. CDB-5 and *Bacillus licheniformis* CDB-12 (Behera et al. 2016). A team led by Okoh has done a lot of studies on optimization of endoglucanase production from different strains of microorganisms. From their studies, it can be understood that optimum production of endoglucanase is solely strain depended. They found that *Bacillus* sp. strain SAMRC-UFH9 (Fatokun et al. 2017) and *Bacillus cereus* SAMRC-UFH1 (Nkohla et al. 2017) preferred 150 rpm whereas *Micrococcus luteus* strain SAMRC-UFH3 (Mmango-Kaseke et al. 2016) chose 50 rpm and *Streptomyces albidoflavus* strain SAMRC-UFH5, 100 rpm (Fatokun et al. 2016) for maximum endoglucanase production.

3.3.1.2. Fermentation media

Media composition is an imperative factor in a fermentation process. The culture medium supplies the microbes with all the essential elements for growth. Mandel and Reese (1957), who were the pioneers of cellulase research, developed the basic medium for cellulase production.

The medium contained different cellulose preparations as the carbon source, organic and inorganic nitrogen sources and trace elements. Since then many different media compositions have been tried by researchers. Generally, in fermentation medium carbon and nitrogen sources play dominant roles as they are directly linked to the production of biomass and metabolites.

3.3.1.2.A. *Carbon sources*

Cellulase being an inducible enzyme the carbon source used plays a vital role in the productivity. A wide range of carbon sources such as CMC, starch, cellulose powder, maltose, mannitol, galactose (Goyal et al. 2014) glucose, sucrose, rhamnose, mannose, sorbitol, raffinose, trehalose (Shankar and Isaiarasu 2011), lactose, fructose (Sethi et al. 2013) arabinose, xylose, cellobiose (Premalatha et al. 2015) have been tested by researchers for cellulase production.

Sethi et al. (2013) observed highest endoglucanase production by *Pseudomonas fluorescens, Bacillus subtilis, E. coli,* and *Serratia marcescens* when glucose was used as carbon source followed by fructose for *B. subtilis, E. coli,* and *S. marcescens* and lactose for *P. fluorescens.* Whereas another strain of *Bacillus subtilis* isolated from cow dung preferred lactose for endoglucanase production followed by sucrose, mannose, starch and glucose (Bai et al. 2012). Xylose was found to be the best carbon source for CMCase production by marine *Bacillus* VITRKHB (Singh et al. 2014a). Researchers like Abou-Taleb et al. (2009), Goyal et al. (2014), Kowsalya and Gurusamy (2014), Lugani et al. (2015), and Premalatha et al. (2015) observed maximum production of endoglucanase

when the medium was supplemented with CMC as carbon source as compared to others. *Bacillus pumilus* EWBCM1 isolated by Shankar and Isaiarasu (2011) showed maximum endoglucanase production when galactose was used as the carbon source.

3.3.1.2.B. *Nitrogen sources*

Nitrogen sources form secondary energy sources for microbes and play a vital role in the cell growth and production of enzymes. Nitrogen is the constituent of amino acids, nucleic acids and coenzymes and comprises about 8-14 % of dry cell mass of bacteria. According to Sethi et al. (2013) the nitrogen and carbohydrate sources in the medium affect the extracellular production of cellulase. The nature and concentration of the source can either stimulate or down-regulate the production of enzymes.

The requirement of nitrogen differs from organism to organism. Over the years different nitrogen sources (organic or inorganic) have been used by researchers for enhancing endoglucanase production. The organic sources used include yeast extract, peptone, urea, beef extract, casein, tryptone, glycine, gelatin, alanine and malt extract. The inorganic sources used commonly are ammonium nitrate, ammonium sulphate, ammonium chloride, ammonium phosphate, ammonium molybdate, potassium nitrate, and sodium nitrate (Shankar and Isaiarasu 2011). Organic nitrogen sources like yeast extract (Acharya and Chaudhary 2012; Kowsalya and Gurusamy 2014) and peptone (Bai et al. 2012; Lugani et al. 2015) were the most favored nitrogen sources for endoglucanase production by different *Bacillus* species. Whereas a combination of both yeast extract

and peptone were required for high endoglucanase production by *Bacillus subtilis* AS3 (Deka et al. 2011), *B. amyloliquefaciens* SS35 (Singh et al. 2014b), and *Bacillus subtilis* BY-2 (Yang et al. 2014). Inorganic nitrogen sources mainly different ammonium salts like ammonium chloride (Liang et al. 2014) ammonium sulphate (Sethi et al. 2013) ammonium nitrate (Sadhu et al. 2014) were found to enhance endoglucanase production in different strains of bacteria. In a study conducted by Goyal et al. (2014), *Bacillus* sp. 313SI was found to produce maximum endoglucanase when ammonium sulphate and ammonium nitrate were used as nitrogen sources under stationary and shaking conditions, respectively.

3.3.1.2.C. *Mineral Salts*

Culture media supplies microbes with all essential elements for growth. Some microorganisms can synthesize most of their cellular components using carbon and nitrogen sources. But most of them require mineral elements for growth and metabolism in addition to carbon and nitrogen. These elements act as cofactors of essential enzymatic reactions occurring in the cell. An optimum concentration of mineral salt increases the utilization of substrate in the culture medium. The mineral salts dissociate into ions in the medium and these ions are thought to affect the catalytic activity and stability of both intracellular and extracellular enzymes (Karim et al. 2015).

Different minerals such as magnesium, phosphorous, potassium, sulphur, calcium and chlorine are essential components of media and are supplemented in the form of their salts. Sreeja et al. (2013) used variety of mineral salts to enhance endoglucanase production from two strains of

Bacillus. *B.altitudinis* showed high enzyme production when magnesium sulphate was used whereas *B. licheniformis* preferred manganous sulphate. Kowsalya and Gurusamy (2014) and Shajahan et al. (2017) reported increased endoglucanase production by *Bacillus subtilis* KG10 and *Bacillus licheniformis* NCIM 5556 respectively, when calcium chloride administered media was used. Metal ions can also have an inhibitory effect on cellulase production. Bahobil et al. (2014) reported an inhibitory effect of metal ions on cellulase production by *B. stearothermophilus* KGKSA40.

3.3.2. Optimization of cellulase production

Enzyme production in microbes is closely controlled and therefore productivity can be improved by ameliorating these controls. Cellulase yield is highly dependent on the complex interaction between various physicochemical parameters. Therefore, for overproduction of enzyme, it is necessary to investigate the significant parameters and find their optimum levels. Thus optimization studies facilitate production of large quantities of enzyme thereby reducing the production cost (Gautam et al. 2011).

Over the years different strategies have been adopted by researchers for optimization studies. The traditional OFAT approach involves changing one factor while keeping the others at constant levels. Although the method is simple and useful for screening, it has few limitations: it determines the effect of only one variable at a time and does not consider the complex interaction among them. Also, the method is time consuming, tedious and requires a number of experiments to find the

optimum levels (Zambare and Christopher 2011). In spite of these drawbacks OFAT method has been widely employed for cellulase optimization in recent years. Researchers such as Acharya and Chaudhary (2012), Kumar et al. (2012b), Lugani et al. (2015) and Hussain et al. (2017) were successful in enhancing the endoglucanase production from various strains of *Bacillus* by optimizing different environmental and nutritional factors via OFAT method. The major parameters that were considered for optimization were temperature, pH, incubation time, inoculum concentration, media volume, agitation, and nitrogen and carbon sources.

Design of Experiments or DOE is an alternative technique used to address the limitations of OFAT method. It was developed by Sir Ronald Aylmer Fisher in 1920's to study the effect of multiple variables simultaneously (Fisher 1949). DOE technique is a statistical tool that analyses the process inputs that have a significant effect on the desired output and finds the level of these inputs to achieve the desired output. It uses a systematic method to determine the optimal process parameters to attain a required product with fewer testing trails. Several DOE techniques are available to the experimental designer and the choice depends on the aim of the experiments.

Generally, DOE is carried out in four stages: planning, screening, optimization and verification. Planning the experiment is the first stage and careful planning will avoid error in the execution of the experiment. In an experiment, there may be many factors influencing the desired output. Screening, which is the second stage, reduces the number of factors by identifying the key factors essential to obtain the required

result. This reduction helps in focusing the improvement studies on the most vital factors. It also suggests the optimal range of the factors which can further be optimized to determine the best level. The main methods used for screening are general factorial design, full and fractional factorial design and Plackett-Burman design. When a search for significant variables that contribute to the final product quality is demanding, factorial design (to eliminate some independent variables that are not statistically significant) is used (Granato and de Araújo Calado 2014). A fractional or full factorial design provides complete information but requires a lot of experiments which make it impracticable when large numbers of variables are to be studied. For a rough estimate of main effects, Plackett-Burman design (PBD) is the most preferred option. The Plackett-Burman design is a two-level fractional factorial design, which allows screening and selection of most significant variables from among a large number of variables. However, it does not consider the interactions between the variables.

Next stage involves optimization of the selected vital components. The techniques used for optimization can be roughly classified into four categories: factorial design, response surface methodology (RSM), Taguchi design, and mixture experiments. Among these RSM is the most widely used method (Myers et al. 2016). This design was developed by Box and Wilson (1951). This technique enables to evaluate the optimal level of each variable, their interactions with other variables and their effect on product yield (Ali et al. 2013). For RSM purposes, there are two quadratic factorial designs: central composite design and Box-Behnken design. Box-Wilson Central Composite Design, commonly called as

central composite design (CCD), is the most efficient and popular RSM design. The last stage of DOE is validation which involves performing few experiments at the predicted optimal levels in order to confirm the results of optimization.

Plackett-Burman Design for screening and central composite design for optimization have been successfully used to enhance the production of various enzymes like cellulase (Thakkar and Saraf 2014), amylase (Cotârlet and Bahrim 2012), protease (Saxena and Singh 2014), gelatinolytic enzyme (Sai-Ut et al. 2014), xylanase (Chaturvedi et al. 2015), chitinase (Warda et al. 2016) etc. Improvement of endoglucanase production by statistical methods has been attempted by many researchers. Parkhey et al. (2016) observed a 2.8 fold increase in endoglucanase production by *Ochrobactrum haematophilum* using this method. They used the following combination of parameters: CMC (4.76 %) yeast extract (2.03 %), pH (6.3), and temperature (44.2 °C) for maximum enzyme production. Recently, endoglucanase production from many bacteria belonging to *Bacillus* spp. has been optimized by a combination of PBD and CCD (Table 3.1).

Table 3.1: Optimization of endoglucanase production by *Bacillus* spp. using Plackett – Burman and central composite design

Strains	Software used	Factors screened (g/l)	Selected components and concentration (g/l)	Fold increase	Reference
Bacillus subtilis AS3	Minitab	CMC- 10 Peptone - 5 yeast extract - 5 K_2HPO_4-1 $MgSO_4 \cdot 7H_2O$ - 0.25 $FeSO_4 \cdot 7H_2O$- 0.25 $MnCl_2 \cdot 4H_2O$- 0.5	CMC – 18 Peptone–8 Yeast extract 4.79	6	Deka et al. (2011)
Bacillus amyloliquefaciens SS35	Minitab	CMC-10 yeast extract -5 peptone - 5 K_2HPO_4 - 1 $MgSO_4 \cdot 7H_2O$ - 0.2 NaCl -1	CMC -18.05 Yeast extract - 8 peptone - 2	3	Singh et al. (2014b)
Bacillus amyloliquefaciens MBAA3	Design expert	CMC Sucrose Yeast extract Peptone K_2HPO_4 $MgSO_4$ Temperature pH	CMC -1.84 $MgSO_4$ - 0.275 pH - 8.5	6.81	Thakkar and Saraf (2014)
Bacillus licheniformis NCIM 5556	Minitab and design expert	CMC yeast extract $CaCl_2 \cdot 6H_2O$ Tween-20 pH temperature inoculum size	CMC -19.21 $CaCl_2 \cdot 6H_2O$- 25.06 mg/l Tween-20 - 2.96 ml/l temperature - 43.35 °C	3	Shajahan et al. (2017)

Response surface methodology has been extensively and effectively used to optimize selected parameters and improve product yield. Kazemi et al. (2014) used Box–Behnken design to optimize endoglucanase production by *Bacillus* sp. BCCS A3. Initially, the carbon and nitrogen sources were selected, while keeping other components at a

constant level. Later the selected sources which were CMC and tryptone were optimized along with pH. Maximum endoglucanase production was obtained using the following composition: CMC 0.75 %, tryptone 0.1 %, and pH 9.0. Shankar and Isaiarasu (2011), used galactose (1.0 g/l), malt extract (0.5 g/l) and incubation time of 72 h to obtain maximum enzyme yield from *Bacillus pumilus* EWBCM1. Singh et al. (2014a) reported maximum CMCase production by *Bacillus* VITRKHB after 24 h incubation using media containing xylose 5.0 %, beef extract 6.9 %, NaCl 1.17 % at pH 7.83 and temperature 25.84 °C. The optimized combination of variables for maximum endoglucanase production by *Enhydrobacter* sp. ACCA2 was determined as 1.5 % CMC, 1.45 % peptone, 0.044 % NaCl, 0.031 % $MgSO_4$, 0.045 % $(NH_4)_2SO_4$, and 0.15 % K_2HPO_4 (Premalatha et al. 2015).

3.4. MATERIALS AND METHODS

3.4.1. Microorganism and culture condition for endoglucanase production

The bacterium *Bacillus subtilis* MU S1 isolated from Eravikulam National Park of Munnar wildlife division was subjected to optimization studies. The seed culture was prepared by inoculating a loopful of the culture into 50 ml unoptimized medium containing following components in g/l: CMC (10.0), NaCl (6.0), $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (0.5), K_2HPO_4 (0.5), $MgSO_4$ (0.1), $CaCl_2$ (0.1), $NaNO_3$ (0.1) and Yeast extract (1.0) at pH 7.0. The culture was incubated in a shaker incubator at 37 °C. One percent of the overnight grown culture (adjusted to a McFarland standard of 1.0, approximately $3x10^8$ CFU/ml) was used as the seed for

endoglucanase production using the unoptimized medium at conditions mentioned above. After 24 h incubation, the supernatant collected following centrifugation at 10,000 g for 10 min was used to analyze enzyme activity. The endoglucanase assay was performed and activity calculated as detailed in section 2.4.5 of chapter 2.

3.4.2. Optimization of physical parameters by OFAT design

The temperature and agitation speed for endoglucanase production were optimized by OFAT method. For optimizing temperature, the unoptimized medium was inoculated with seed culture and incubated at varying temperatures (30 °C, 40 °C and 50 °C) in a shaker incubator. After 24 h the culture was centrifuged and the cell free supernatant was used as the crude enzyme for endoglucanase assay. The optimal agitation speed was determined by inoculating and incubating medium at different agitation speeds (50, 100, 150 and 200 rpm) at optimized temperature. Endoglucanase assay was performed in duplicate with crude enzyme obtained from 24 h culture. The medium components were optimized by growing the organism under the optimized physical conditions.

3.4.3. Medium optimization by statistical method

3.4.3.1. *Plackett-Burman Design for screening of significant medium components*

The most important variables that significantly influence CMCase production was selected by Plackett-Burman design using the statistical software package MINITAB (Release 16, PA, USA). A total of ten parameters were screened and each parameter was examined at its low

level (−1) and high level (+1) (Table 3.2). PBD is based on the first order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i$$

where Y is the response (CMCase activity), β_0 is the intercept of the model, β_i is the linear coefficient, and X_i is the level of the independent variable. This design does not consider the interaction among variables and a linear approach is considered to be sufficient for screening. The main effects of such a design are calculated as the difference between the average of measurements made at the high level (+1) of the factor and the average of measurements at the low level (−1) .

Table 3.2: Medium components and their variables used in Plackett-Burman design

Nutrient code	Nutrients (g/l)	Low (-1)	High (+1)
A	CMC	2	18
B	Yeast extract	0.5	10.5
C	NaCl	2	14
D	$(NH_4)_2SO_4$	0.5	2.5
E	KH_2PO_4	0.05	2.05
F	K_2HPO_4	0.05	0.45
G	$MgSO_4$	0.01	0.21
H	$CaCl_2$	0.005	0.405
I	$NaNO_3$	0.005	0.805
J	pH	5.0	7.0

The ten variables were screened in 20 experimental runs. Averages of endoglucanase activity obtained from duplicate experiments were taken as the response (Table 3.4). Regression analysis was performed to determine the factors that influence enzyme production. The

factors which were significant at or above 95 % level ($p < 0.05$) were selected and later optimized by central composite design.

3.4.3.2. *Central composite design*

After identifying the significant variables for CMCase production by PBD, response surface methodology using CCD was employed to determine the optimal levels of these variables. A 3-factor-5-level design was used and five coded levels (-α, -1, 0, +1, + α) were assigned to each factor (Table 3.3). Alpha is the extended level with the value of $(2)^{3/4}$ = 1.682. A 2^3 full-factorial CCD experimental design containing three significant medium components (CMC, yeast extract and NaCl) at five coded levels was generated using the statistical software package "Design Expert 7" (Stat Ease Inc., Minneapolis, USA). The experimental design comprised of 20 runs ($=2^k + 2k + n_0$), where 'k' is the number of independent variables and n_0 is the number of replicate runs at the center point of the variables. All experiments were carried out in duplicate and the averages of the CMCase activity were taken as the response (Table 3.6).

Table 3.3: Experimental range and levels of independent variables used for central composite design

Variables	Components	Levels				
		- α	**-1**	**0**	**+1**	**+ α**
A	CMC	2	5.24	10	14.76	18
B	Yeast extract	0.5	2.53	5.5	8.47	10.5
C	NaCl	2	4.43	8	11.57	14

The coded values of independent variables were calculated using following equation

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad i = 1, 2, 3, \ldots, k$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the value of X_i at the center point, and ΔX_i is the step change. The experimental data were analyzed using multiple regression analysis. The following second-order polynomial equation was applied to determine the relationships and interrelationships of the variables

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{23} BC + \beta_{13} AC$$

where Y is the predicted response, β_0 is the intercept, β_1, β_2, β_3 are linear coefficients, β_{11}, β_{22}, β_{33} are quadratic coefficients, and β_{12}, β_{23}, β_{13} are the interaction coefficients, A,B and C are the independent variables studied.

The results of CCD were analyzed using ANOVA (analysis of variance) test of Design Expert 7, to determine the significance of each term in the equations fitted and to estimate the goodness of fit of the model. The fitted polynomial equation was graphically represented in the form of three-dimensional surface plots. These graphs display the main and interactive effects of the selected factors on endoglucanase production.

3.4.3.3. *Validation of the model*

The statistical model for the production of endoglucanase was validated by performing experiments under five different sets of predicted conditions (Table 3.8). The combination showing highest activity was chosen as the optimized medium. To determine the effect of $MgSO_4$ and $NaNO_3$, endoglucanase production of the strain in optimized medium containing $MgSO_4$ (0.01 g/l) and $NaNO_3$ (0.005 g/l) was also determined under optimized conditions.

3.4.4. Overall optimization

To find the overall enhancement in endoglucanase production after OFAT and statistical optimization, the strain was grown in optimized media under the optimal level of physical conditions. The fold increase in enzyme activity after optimization was determined by comparing the activity obtained under unoptimized and optimized conditions.

3.4.5. Growth curve analysis

In order to determine the relationship between the time course of bacterial growth and endoglucanase production in optimized medium, a growth curve analysis was performed. *B. subtilis* MU S1 was grown in optimized medium at 40 °C. Bacterial growth and activity were measured by collecting samples after every 2 h, till the stationary phase is reached.

3.5. RESULTS

3.5.1. Production of endoglucanase under unoptimized conditions

In the study, endoglucanase was produced from *Bacillus subtilis* MU S1 by fermentation in the unoptimized medium at 37 °C in a shaker incubator. After 24 h incubation, an activity of 179.06 ± 3.2 U/ml was obtained. The production of endoglucanase from strain MU S1 was improved by optimizing a range of physical and nutritional parameters by OFAT and statistical methods respectively.

3.5.2. Optimization of physical parameters for endoglucanase production

The external factors that influence endoglucanase production: temperature and agitation speed were optimized by OFAT method. In *Bacillus subtilis* MU S1 the maximum endoglucanase production (187.08 ± 2.8 U/ml) was observed at 40 °C. The production decreased at 30 °C followed by 50 °C (Figure 3.1 A). To determine the optimum agitation speed the strain was incubated at four different agitation speeds at 40 °C. From Figure 3.1 B it is evident that the optimum agitation speed for strain MU S1 was 150 rpm (191.44 ± 3.8 U/ml). Further increase in agitation rate decreased the enzyme production.

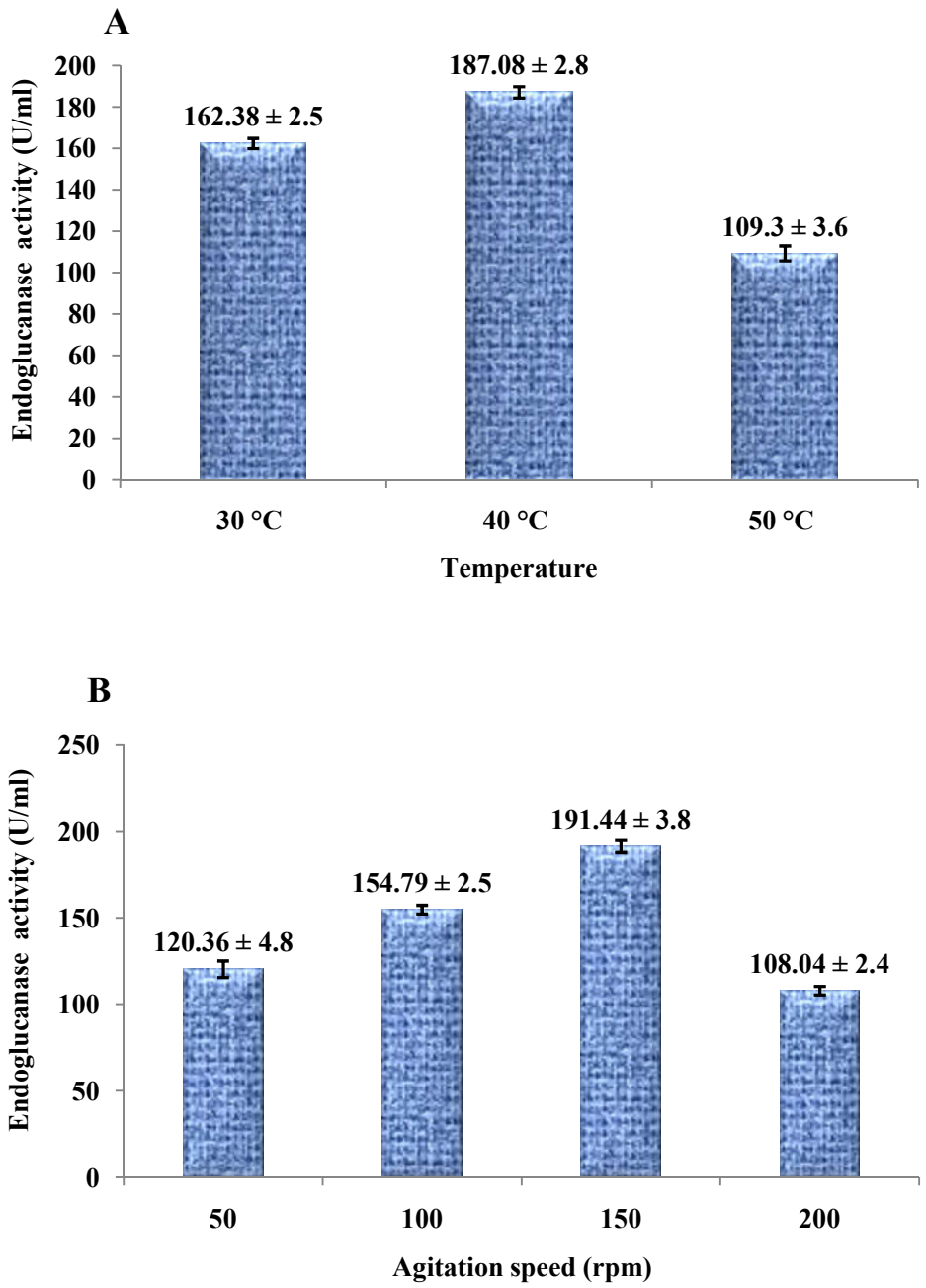


Figure 3.1: Optimization of physical parameters **(A)** temperature **(B)** agitation speed

3.5.3. Medium optimization for endoglucanase production

3.5.3.1. *Screening the significant medium components using Plackett-Burman Design*

Using PBD the most significant factors were screened from among ten components in 20 experimental runs. As indicated in Table 3.4, the CMCase activity of *Bacillus subtilis* MU S1 showed a wide variation from 8.30 to 500.80 U/ml. The important variables influencing endoglucanase production were selected based on p-value < 0.05 and confidence level $\geq$ 95 %. The components CMC, yeast extract, NaCl, pH, $MgSO_4$ and $NaNO_3$ showed $p < 0.05$. Among them CMC (p=0.00), yeast extract (p=0.00), NaCl (p=0.003) and pH (p=0.003) showed significant positive effect whereas $MgSO_4$ (p=0.006) and $NaNO_3$ (p=0.012) showed negative effect. The confidence level was approximately 100 for all the five factors. The Pareto chart (Figure 3.2) gives a pictorial representation of the significant factors. The bars that cross the reference line (t value 2.26) are statistically significant at 0.05 levels. However, the chart does not indicate the nature of effect of the factors. The overall regression coefficient for Placket-Burman design was R^2= 97.68 % with adjusted R^2 = 95.11 % and predicted R^2 = 88.56 %. The values of first order model coefficient, t- value, p-value and confidence levels of all ten variables are represented in Table 3.5.

Table 3.4: Plackett-Burman design for ten variables along with the observed results

Run	CMC	Yeast extract	NaCl	$(NH_4)_2SO_4$	KH_2PO_4	K_2HPO_4	$MgSO_4$	$CaCl_2$	$NaNO_3$	pH	Endoglucanase activity (U/ml)
1	1	-1	-1	-1	-1	1	-1	1	-1	1	190.48
2	1	-1	-1	1	1	-1	1	1	-1	-1	8.30
3	1	1	-1	1	1	1	-1	-1	-1	1	500.80
4	1	1	1	-1	-1	1	1	-1	1	1	440.32
5	-1	-1	1	-1	1	-1	1	1	1	1	10.81
6	-1	-1	-1	1	-1	1	-1	1	1	1	15.30
7	1	-1	1	-1	1	1	1	1	-1	-1	150.83
8	1	-1	1	1	1	1	-1	-1	1	1	170.69
9	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	10.53
10	1	-1	1	1	-1	-1	-1	-1	1	-1	130.40
11	-1	-1	1	1	-1	1	1	-1	-1	-1	16.34
12	-1	1	1	1	1	-1	-1	1	1	-1	320.16
13	1	1	-1	-1	1	1	-1	1	1	-1	300.64
14	1	1	1	1	-1	-1	1	1	-1	1	444.50
15	-1	1	-1	1	-1	1	1	1	1	-1	100.40
16	-1	-1	-1	-1	1	-1	1	-1	1	1	9.86
17	1	1	-1	-1	-1	-1	1	-1	1	-1	330.42
18	-1	1	1	-1	-1	-1	-1	1	-1	1	370.62
19	-1	1	1	-1	1	1	-1	-1	-1	-1	382.63
20	-1	1	-1	1	1	1	1	-1	-1	1	278.21

Table 3.5 Statistical analysis of Plackett-Burman design for ten variables

Term	Effect	Coef	SE Coef	T-Value	P-Value	Confidence level (%)
Constant		209.11	8.39	24.92	0.000	100
CMC	115.25	57.63	8.39	6.87	0.000	100
Yeast extract	275.52	137.76	8.39	16.42	0.000	100
NaCl	69.24	34.62	8.39	4.13	0.003	99.7
$(NH_4)_2SO_4$	-21.20	-10.60	8.39	-1.26	0.238	76.2
KH_2PO_4	8.36	4.18	8.39	0.50	0.630	37.0
K_2HPO_4	-9.06	-4.53	8.39	-0.54	0.603	39.7
$MgSO_4$	-60.23	-30.11	8.39	-3.59	0.006	99.4
$CaCl_2$	-35.82	-17.91	8.39	-2.13	0.062	93.8
$NaNO_3$	-52.42	-26.21	8.39	-3.12	0.012	98.8
pH	68.09	34.05	8.39	4.06	0.003	99.7

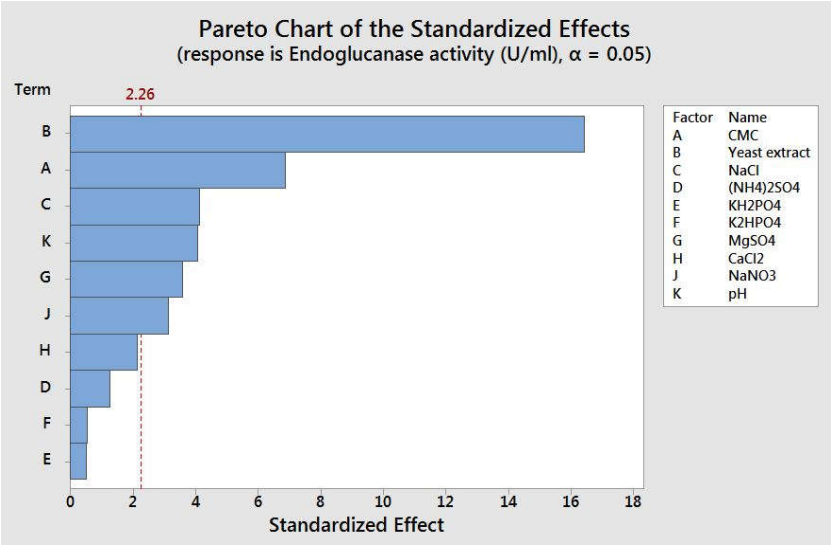


Figure 3.2: Pareto chart showing effect of medium components on endoglucanase activity

3.5.3.2. *Optimization using central composite experimental design*

Among the six factors selected in PBD, the factors which showed negative effect viz $MgSO_4$ and $NaNO_3$ were eliminated from further optimization, and CCD was carried out with CMC, yeast extract and NaCl maintaining pH at neutral, at which the strain exhibited maximum enzyme production. A full factorial CCD was performed with these 3 factors and the results were recorded in Table 3.6. The predicted endoglucanase activity was calculated using the second order polynomial equation

$$\begin{aligned}\text{Endoglucanase activity (U/ml)} = {} & + 95.48885 + 37.85381A + 34.48640B - 1.29548C \\ & + 3.43451AB - 0.78909AC + 1.96564BC \\ & - 2.07696A^2 - 5.28523B^2 - 0.54335C^2\end{aligned}$$

where A, B and C are concentrations of CMC, yeast extract and NaCl in g/l. The statistical significance of the second order polynomial equation was evaluated by the *F* test and the result of ANOVA was recorded in Table 3.7. The model was significant as indicated by "model *F*-value" of 50.29 and *p*-values less than 0.05. In the present work, linear terms A, B, C the square effect of A, B and combinations of AB and BC were found to be significant for endoglucanase activity. The insignificant "lack of fit *F*-value" (2.41) implies that the predicted results were in a good fit. The R^2 value (multiple correlation coefficient /determination coefficient) obtained was 0.9784. The predicted R^2 of 0.8747 was also in reasonable agreement with adjusted R^2 of 0.9589 which indicates that the model fits very well to the experimental data. This was confirmed by the predicted endoglucanase activity which matches well with the observed activity. The adequate precision of the present analysis was 23.672.

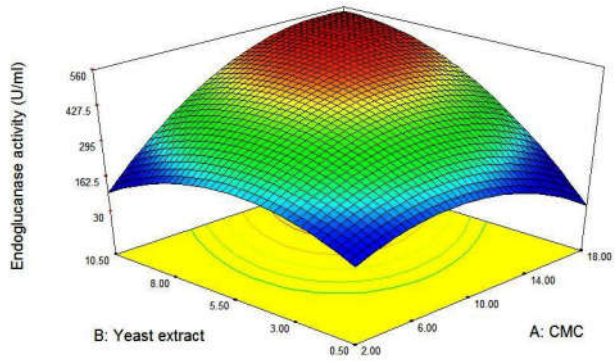
Table 3.6: Full factorial central composite design matrix with actual values of variables and the observed and predicted response.

Run	CMC	Yeast extract	NaCl	Endoglucanase activity (U/ml)	
				Observed	Predicted
1	14.76	2.53	4.43	350.26	337.44
2	10.00	5.50	2.00	492.13	486.10
3	5.24	8.47	4.43	324.28	341.22
4	5.24	2.53	11.57	268.31	257.74
5	10.00	5.50	8.00	479.33	463.25
6	14.76	8.47	11.57	506.40	514.18
7	14.76	8.47	4.43	536.32	549.79
8	14.76	2.53	11.57	232.50	218.46
9	2.00	5.50	8.00	268.03	259.19
10	18.00	5.50	8.00	396.63	401.46
11	10.00	5.50	8.00	465.26	463.25
12	5.24	8.47	11.57	343.48	359.24
13	10.00	5.50	8.00	471.32	463.25
14	5.24	2.53	4.43	328.06	323.08
15	10.00	5.50	8.00	456.53	463.25
16	10.00	5.50	14.00	399.26	401.27
17	10.00	5.50	8.00	434.32	463.25
18	10.00	5.50	8.00	472.04	463.25
19	10.00	0.50	8.00	172.19	199.02
20	10.00	10.50	8.00	494.06	463.21

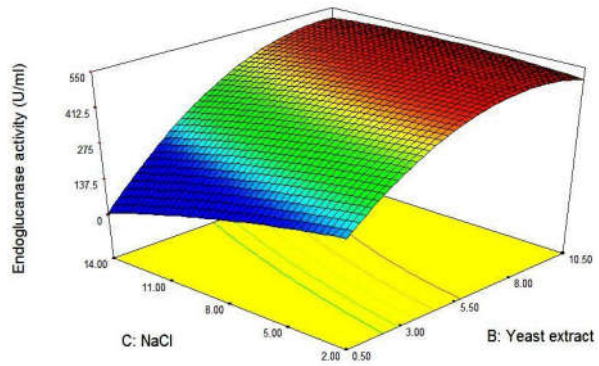
Table 3.7: Analysis of variance (ANOVA) of response surface quadratic model for the production of endoglucanase by *Bacillus subtilis* MU S1

Source	Sum of squares	df	Mean square	*F* value	*p*- value Prob > F	
Model	1.988E+005	9	22083.76	50.29	< 0.0001	significant
A-CMC	24431.30	1	24431.30	55.63	< 0.0001	
B-Yeast extract	84252.06	1	84252.06	191.85	< 0.0001	
C-NaCl	8686.03	1	8686.03	19.78	0.0012	
AB	18873.33	1	18873.33	42.98	<0.0001	
AC	1434.60	1	1434.60	3.27	0.1008	
BC	3477.36	1	3477.36	7.92	0.0184	
A^2	31829.57	1	31829.57	72.48	< 0.0001	
B^2	31449.98	1	31449.98	71.61	< 0.0001	
C^2	689.26	1	689.26	1.57	0.2388	
Residual	4391.58	10	439.16			
Lack of Fit	3104.57	5	620.91	2.41	0.1780	not significant
Pure Error	1287.02	5	257.40			
Cor Total	2.031E+005	19				

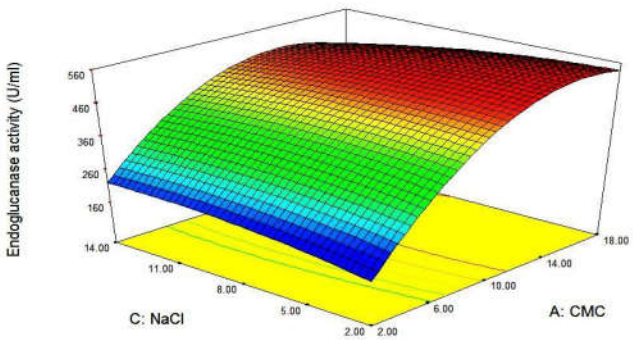
The effect of the interaction of variables on endoglucanase production was studied by three-dimensional surface curves against two independent variables while the other independent variable was maintained at its constant level. These surface plots demonstrate the individual and interactive effect of the media components and can be used to predict the optimal value of different variables. Three response surface plots were obtained by considering all the possible combinations (Figure 3.3). The 3D response surface plot shown in Figure 3.3A depicts the interaction between CMC and yeast extract. The endoglucanase activity increases with increasing concentration of both the components, however, when the concentrations reach beyond the middle value the activity tends to decline. The plot shows strong interaction between the components which was confirmed by p-value < 0.0001. Figure 3.3B shows the interaction between yeast extract and NaCl. In case of yeast extract the surface curvature is ascending indicating an increase in activity, but beyond about 8 g/l the activity decreases. For NaCl, no much surface curvature was seen but the activity tends to decrease with higher concentration. Figure 3.3C describes the interaction between CMC and NaCl. From the graph, it is evident that there is little or no interaction between the two components. High level of CMC and almost middle level of NaCl was most optimum for high endoglucanase production.



(A)



(B)



(C)

Figure 3.3: Response surface graph showing interaction effects between concentrations of selected variables **(A)** CMC and yeast extract **(B)** NaCl and yeast extract **(C)** CMC and NaCl

3.5.3.3. *Validation of the model*

In order to verify the adequacy of statistical analysis and quadratic model, a set of experiments were performed in duplicate using the combinations suggested by the software (Table 3.8). The maximum endoglucanase activity predicted by the model using the optimum concentration (13.46 g/l CMC, 8.38 g/l yeast extract and 6.31 g/l NaCl at pH 7.0) was 541.05 U/ml. This was in agreement with the experimental yield of 566.62 U/ml, which verifies the validity of the model and existence of optimal points. The presence of $MgSO_4$ and $NaNO_3$ in the optimized medium decreased the activity to 516.23 U/ml.

Table 3.8: Validation of the model

Run	CMC	Yeast extract	NaCl	Endoglucanase activity (U/ml)	
				Observed	Predicted
1	13.46	8.38	6.31	566.62	541.05
2	13.17	8.32	5.15	551.23	540.81
3	13.98	8.45	7.72	528.54	539.21
4	14.02	7.55	5.23	546.08	538.71
5	13.26	7.58	4.92	519.78	537.46

3.5.4. Overall optimization

After OFAT and statistical optimization the improvement in endoglucanase production was determined. There was an overall 3.2 fold increase in endoglucanase after optimization.

3.5.5. Growth curve analysis

The growth curve analysis showed that growth and endoglucanase activity were related to each other. Highest activity was observed in the late log phase, thereafter the activity reduced slightly (Figure 3.4).

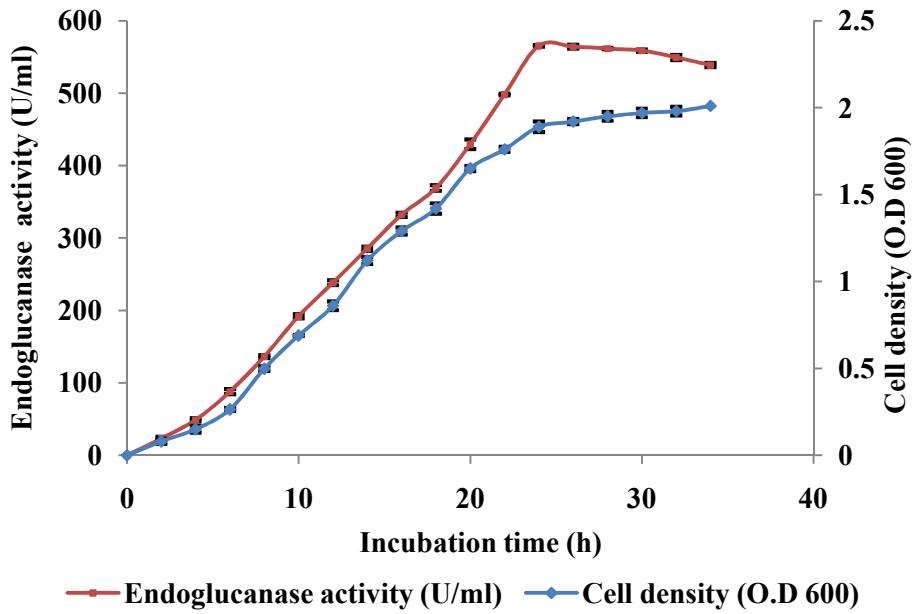


Figure 3.4: Endoglucanase production profile and growth curve of *B.subtilis* MU S1 in optimized medium

3.6. DISCUSSION

Biotechnological processes are generally highly dependent on production parameters and optimization of these parameters enhances the yield, improves reproducibility and also reduces the cost of production. There is no general medium for enzyme production by different microbial strains. Cell growth and enzyme production are greatly influenced by the

chemical composition of culture medium and environmental factors. Moreover, each strain has its own idiosyncratic physicochemical and nutritional requirements for growth and secretion of the enzyme (Reddy et al. 2008). In order to improve the enzyme production, the physical parameters and medium components were optimized. Lastly, the fold increase was monitored.

The physical parameters (temperature and agitation speed) for endoglucanase production were optimized using the traditional method of OFAT. Temperature is a critical parameter which influences extracellular enzyme production by changing the physical properties of the cell membrane. In case of *Bacillus subtilis* MU S1 the maximum endoglucanase production (187.08 ± 2.8 U/ml) was observed at 40 °C. Lower activity was observed at 30 °C and 50 °C. The studies of Immanuel et al. (2006) showed that the microorganisms belonging to *Cellulomonas*, *Bacillus,* and *Micrococcus* sp. produced maximum endoglucanase at 40 °C and neutral pH. Ray et al. (2007) also observed maximum cellulase yield at 40 °C by *Bacillus subtilis*. Agitation speed is another important culture parameter that maintains homogenous condition and disperses dissolved oxygen, thus helps in enhancing both substrate utilization and microbial activity. The optimum agitation speed for strain MU S1 was 150 rpm (191.44 ± 3.8 U/ml). Enzyme production decreased at lower and higher speeds. Cell death and enzyme inactivation due to shearing effect may be the reason for low activity at higher agitation speeds. Dash et al. (2015) tried agitation speed between 100-180 rpm and found that 150 rpm was optimum for enzyme production by *Bacillus subtilis* BI19. The

optimum temperature and agitation speed for further experiments were fixed based on OFAT results.

Media components and their interactions play vital role in extracellular enzyme production. Initially, the influence of ten variables on endoglucanase production was studied by Plackett- Burman method. Six variables viz. CMC, yeast extract, NaCl, $MgSO_4$, $NaNO_3$ and pH that significantly affect endoglucanase production were selected based on the *p* and *t* values. These factors had *p*-value < 0.05 and *t* value greater than 2.26. The sign of *t* value indicates the type of effect that is either positive or negative. Among the factors $MgSO_4$ and $NaNO_3$ were indicated to have a negative effect on endoglucanase production.

Among the selected components CMC acts as the sole carbon source. Carboxymethyl cellulose is reported as the best carbon source for endoglucanase production by many *Bacillus* spp. (Abou-Taleb et al. 2009; Goyal et al. 2014). Niranjane et al. (2007) also found that CMC was a superior carbon source for endoglucanase production when compared with cellulose. Among the nitrogen sources, yeast extract was shown to have an immense influence on enzyme production. The presence of external nitrogen source is essential in fermentation media during extracellular enzyme production for effective utilization of carbohydrates. Organic nitrogen sources are preferred for cellulase production compared to inorganic sources. In our medium yeast extract, $(NH_4)_2SO_4$ and $NaNO_3$ acted as nitrogen sources and among them, yeast extract was found to have an immense influence on enzyme production. Abou-Taleb et al. (2009) has also reported yeast extract to be the best nitrogen source. Sodium nitrate was also selected but was found to have negative influence

on endoglucanase production. The results suggest that organic nitrogen was more suitable for endoglucanase production than inorganic source. This was in accordance with the results of Ray et al. (2007) who reported that *Bacillus subtilis* preferred organic nitrogen sources. Higher enzyme production in the presence of organic nitrogen may be attributed to the vitamins and growth precursors present in it. Acharya and Chaudhary (2011), reported that *Bacillus licheniformis* WBS1 and *Bacillus* sp. WBS3 did not show any detectable CMCase activity when $(NH_4)_2SO_4$ and $NaNO_3$ was used as the sole source of nitrogen.

Sodium chloride and $MgSO_4$ were also found to influence endoglucanase production; NaCl was positively significant whereas $MgSO_4$ showed negative effect. Presence of both these components in the medium is thought to play a crucial role in initial cell growth. Researchers have demonstrated a positive effect of NaCl on endoglucanase production by different *Bacillus* species (Shankar and Isaiarasu 2011; Kowsalya and Gurusamy 2014). Whereas in case of $MgSO_4$ there are contrasting reports. Singh et al. (2014b) recorded a negative impact of $MgSO_4$ on endoglucanase activity whereas Thakkar and Saraf (2014) recorded a positive effect with confidence level > 95 %. The production of endoglucanase by *Bacillus* sp. JS14 was enhanced by the addition of NaCl and $MgSO_4$ (Singh and Kaur 2012). Another factor that was selected was initial pH of the medium. The pH of the growth medium strongly influences many enzymatic reactions by affecting the transport of chemical products and enzymes across the cell membrane (Liang et al. 2010). *Bacillus subtilis* MU S1 showed high endoglucanase production with initial pH 7.0. Optimum growth pH near neutrality has been reported

earlier for *Bacillus subtilis* KG10 (Kowsalya and Gurusamy 2014) and *Bacillus subtilis* CY5 (Ray et al. 2007). Amongst the remaining components, KH_2PO_4 had positive *t* value while $(NH_4)_2SO_4$, K_2HPO_4, $CaCl_2$ showed a negative value, but none of them was found to significantly affect endoglucanase production by *Bacillus subtilis* MU S1.

In the next stage, the components that positively affect endoglucanase production were optimized using the central composite design of RSM. The optimal levels of CMC, yeast extract and NaCl for maximum endoglucanase production were determined, maintaining the pH of the medium at neutral. From the results of ANOVA which include high *F*-value (50.29), low *p*-value (less than 0.05), non-significant lack of fit (2.41), determination coefficient close to 1 (R^2 of 0.9784), and adequate precision greater than 4 (23.672), it was confirmed that the proposed model was valid. The R^2 value of 0.9784 revealed that the model could explain 97.84 % variation in the response. Normally the R^2 value is between 0 and 1, and closer the value to 1 stronger the model and better it predicts the response. Another index for the fitness of model is Adequate Precision (AP) that measures the signal-to-noise ratio. A ratio greater than 4 is wanted for a good model. The ratio obtained for the present analysis was 23.672 which indicates an adequate signal. Thus this model can be used to navigate the design space identified by central composite design. Among the different combinations of the selected components predicted by the software, best five were selected and validated. The close similarity between the predicted and observed endoglucanse activity proves the efficiency of the model.

The direct visualization of interaction between variables and their effect on the response is possible with the help of 3D surface plots. The Figure 3.3 clearly indicates that maximum endoglucanase activity was observed at a high concentration of CMC and yeast extract and low concentrations of NaCl. Cellulases are inducible enzymes and CMC is known to have an inducing effect on cellulase production. Sadhu et al. (2014) has also reported the importance of CMC as a substrate for endoglucanase production by *Bacillus* sp. Similar observations were made by Deka et al. (2011) and Goyal et al. (2014). These facts can be justified by the report that enzymes involved in substrate degradation are generally inducible and produced only when the corresponding substrate is present in the nutrient media. One mole of CMCase production requires a large quantity of nitrogen, therefore nitrogen sources like yeast extract play a vital role in endoglucanase production. High yeast extract concentrations were found to be optimal for CMCase production by many *Bacillus* spp. (Goyal et al. 2014). The Figure 3.3B and C indicate that sodium chloride was essential but only at a lower concentration.

The cultivation of strain MU S1 in medium containing CMC, yeast extract and NaCl at pH 7.0, temperature 40 °C and agitation speed of 150 rpm significantly improved enzyme production. The optimal levels of the components were CMC 13.46 g/l, yeast extract 8.38 g/l and NaCl 6.31 g/l. The lower endoglucanase activity in the presence of $MgSO_4$ and $NaNO_3$ confirmed the output of PBD. The endoglucanase activity under unoptimized condition was 179.06 U/ml, however, after optimization, the overall activity increased by 3.2 fold as compared to the unoptimized condition. Medium components made a higher contribution towards the

improvement of enzyme production compared to physical parameters. Other researchers, Deka et al. (2013) and Singh et al. (2014b) have also opined that concentration of medium components is the foremost factor for endoglucanase production.

On growth curve analysis it was observed that the endoglucanase activity increased with increase in biomass and reached maximum in late log phase after approximately 24 h of incubation. As the strain entered stationary phase the activity started decreasing. Similar observation was made by Shajahan et al. (2017).

3.7. CONCLUSION

In the present study endoglucanase production from *Bacillus subtilis* MU S1 was enhanced by optimizing the physical parameters and medium components. Successive optimization by OFAT and statistical methods gave an overall 3.2 fold increase in endoglucanase production. A statistical approach for optimization reduced the number of parameters and experiments required for the production of enzymes thus reducing the production cost. The data reveals the basic requirement of the strain for high production of endoglucanase with minimum media components.

SREENA C. P. “IMPROVEMENT OF BIOCATALYTIC EFFICACY OF ENDOGLUCANASE FROM INDIGENOUS STRAIN, BACILLUS SUBTILIS MU S1 AND ITS APPLICATION IN SACCHARIFICATION OF AGRO-WASTES”. THESIS. DEPARTMENT OF LIFE SCIENCES, UNIVERSITY OF CALICUT, 2018.

3.1. INTRODUCTION

Endo-β-1,4-glucanase or CMCase [EC 3. 2. 1.4] is the primary enzyme of the multienzyme complex –cellulases. It hydrolyses the cellulose chains internally providing new chain ends for other cellulases to act synergistically. Endoglucanase has potential application in many industries like detergent, biofuel, textile, paper recycling, juice extraction and animal feed additives (Bhat 2000). Consequently, there has been growing demand for endoglucanase, especially those with high activity and stability under extreme pH and temperature.

The cost of enzymes is one major factor determining the economics of a biocatalytic reaction. Various factors like temperature, pH, aeration and media constituents are known to influence enzyme production. Both physical and nutritional parameters play a crucial role in enhancing cell growth and thereby accumulation of product (Ibrahim and Elkhidir 2011). Medium constituents contribute about 30-40 % of the production cost of industrial enzymes and thus a considerable reduction in cost can be achieved by optimizing the media constituents. Accordingly, optimization studies (physical and nutritional parameters) are inevitable and forms an essential part of all enzyme research.

Conventionally, one-factor-at-a-time/one-variable-at-a-time (OFAT/ OVAT) approach, which involves changing one factor at a time while keeping the others at constant levels, has been used for media optimization studies. This technique has major disadvantages: it does not take into account the complex interactions among various components, moreover, it is time-consuming and requires a number of experiments to determine the

optimum levels (Zambare and Christopher 2011). These shortcomings can be overcome by using Design of Experiments (DOE) method. This statistical technique allows screening and selection of the factors that have a great influence on the output, followed by optimization of their levels. Full factorial design, fractional factorial design and response surface methodology are the statistical tools commonly used.

The full factorial design gives full information but is laborious as it involves a huge number of experiments making this method impracticable when large numbers of factors are being considered. The Plackett-Burman design is a two-level fractional factorial design, which facilitates the screening and selection of essential parameters and also gives convincing information about all the parameters. As compared to earlier design it is rapid, effective and time-saving (Plackett and Burman 1946). Response Surface Methodology (RSM) is a statistical and mathematical tool used for optimization of selected factors (Myers et al. 2016). This technique helps in finding the optimal levels of each variable, their interaction with each other and the net effect on the output (Ali et al. 2013). Recently, Plackett-Burman Design and RSM have been successfully used to optimize many fermentation media.

3.2. OBJECTIVES

The aim of the present chapter was to identify the optimal levels of critical parameters for enhanced production of endoglucanase by *Bacillus subtilis* MU S1. Physical parameters and medium constituents were optimized using OFAT and statistical techniques respectively.

3.3. REVIEW OF LITERATURE

Production cost is believed to be the major bottleneck in many biotechnology-based industrial processes. Fermentation media contributes a lot to the production cost and thus formulation of a suitable media is of critical importance. The fermentation media affects not only the product yield but also volumetric productivity (Singhal and Swaroop 2013). Cellulase production is influenced by a number of factors like culture conditions – including media components and physical parameters, nature of substrate and also potential of the strain used. However, the ultimate production of the enzyme depends on the interrelationship between these factors (Lynd et al. 2002). Thus, for designing a suitable production media, the fermentation conditions (temperature, pH, agitation speed etc.) and media constituents (carbon, nitrogen, metal ions etc.) must be carefully identified and optimized. Although bacteria are more tolerant to extreme environmental conditions compared to other microorganisms, each species has its own characteristic range of values at which it shows maximum growth and metabolic activity.

3.3.1. Factors affecting endoglucanase production

3.3.1.1. Fermentation conditions

The physicochemical parameters that generally affect extracellular enzyme production are temperature, pH and dissolved oxygen.

3.3.1.1.A. *Temperature*

Incubation temperature is one major factor that affects a variety of cell metabolic processes such as denaturation of protein, enzyme activity

and growth of microbes. According to Jung et al. (1974) temperature affects the specific growth rate and also physiological properties of the strain. They found that the secretion of enzymes is greatly dependent on the growth temperature. The optimization of temperature is thus a crucial factor for higher production of the enzyme.

The optimum incubation temperature for endoglucanase production varies depending on the microorganisms. Sethi et al. (2013) reported 40 °C as optimum temperature for endoglucanase production by *Pseudomonas fluorescens, Bacillus subtilis, E. coli,* and *Serratia marcescens.* The same optimum temperature was recorded for endoglucanase from *Cellulomonas, Bacillus,* and *Micrococcus* sp. by Immanuel et al. (2006). Optimum endoglucanase production at 37 °C was observed for *Paenibacillus curdlanolyticus* B-6 (Waeonukul et al. 2009) and *Paenibacillus polymyxa* (Kumar et al. 2012a). Whereas,Liang et al. (2014) obtained maximum endoglucanase activity after incubation at a temperature below 30 °C by a strain of *P. terrae*. Acharya and Chaudhary (2012) found a temperature of 50 °C as optimum for endoglucanase production by *Bacillus licheniformis* MVS1 and *Bacillus* sp. MVS3.

3.3.1.1.B. *pH*

Bacteria are very sensitive to the hydrogen ion concentration of their environment. The pH of growth medium plays a crucial role in enzyme secretion. The pH change during the growth of microbes affects the stability of the enzyme in the media. Variation in pH may bring about an alteration in charge and shape of the protein, leading to loss of catalytic property.

Cellulase production has been detected over a range of pH. Acidic pH for optimum enzyme production is usually found in fungi whereas bacteria prefer near neutral to slightly alkaline pH for enhanced enzyme production. Initial pH near neutrality was found to be most favorable for *P. curdlanolyticus* B-6 (Waeonukul et al. 2009), *P. polymyxa* (Kumar et al. 2012a), *Bacillus amyloliquefaciens* SS35 (Singh et al. 2014b), *Bacillus subtilis* AS3 (Deka et al. 2013), *Bacillus* sp. (MTCC10046) (Sadhu et al. 2014). While researchers like Kazemi et al. (2014), Liang et al. (2014), Thakkar and Saraf (2014), and Dave et al. (2015) noted high endoglucanase production when the initial pH of the medium was slightly alkaline.

3.3.1.1.C. *Agitation rate*

Agitation rate is an important factor that determines the level of dissolved oxygen in the medium and affects both growth and enzyme production by microorganisms (Jo et al. 2008). The requirement of oxygen varies depending on the microbe. Oxygen is the terminal electron acceptor for oxidative reactions and provides energy for cellular activities. Proper agitation is a must to achieve proper mixing, and mass and heat transfer (Amadi and Okolo 2013). There is a critical concentration of dissolved oxygen below which changes can occur in metabolism pathway. According to Feng et al. (2003) high agitation speed increases the dissolved oxygen and dispersion of macromolecules in the medium and thus contributing to high cell growth and enzyme production. But higher speed causes shearing effect and leads to cell death and enzyme inactivation.

From the literature review, it was clear that agitated condition was preferred over static for cellulase production by most of the bacteria. A study conducted by Hussain et al. (2017) showed that the bacteria *B. megaterium* BMS4, *B. subtilis* BTN7A, and *B. amyloliquefaciens* SA5 produced higher endoglucanase after incubation under shaking condition whereas *Anoxybacillus flavithermus* BTN7B showed higher enzyme activity under static condition. The agitation speed of 120 rpm was found to be favourable for endoglucanase production by *Bacillus subtilis* AS3 (Deka et al. 2013), *Lactococcus lactis* and *Cellulomonas fimi* (Shinde and Sharma 2014), *Bacillus* sp. Y3 (Lugani et al. 2015), and *Bacillus subtilis* K-18 (Irfan et al. 2017). Slightly higher agitation speed (150 rpm) was preferred by *Brucella* sp. CDB-5 and *Bacillus licheniformis* CDB-12 (Behera et al. 2016). A team led by Okoh has done a lot of studies on optimization of endoglucanase production from different strains of microorganisms. From their studies, it can be understood that optimum production of endoglucanase is solely strain depended. They found that *Bacillus* sp. strain SAMRC-UFH9 (Fatokun et al. 2017) and *Bacillus cereus* SAMRC-UFH1 (Nkohla et al. 2017) preferred 150 rpm whereas *Micrococcus luteus* strain SAMRC-UFH3 (Mmango-Kaseke et al. 2016) chose 50 rpm and *Streptomyces albidoflavus* strain SAMRC-UFH5, 100 rpm (Fatokun et al. 2016) for maximum endoglucanase production.

3.3.1.2. Fermentation media

Media composition is an imperative factor in a fermentation process. The culture medium supplies the microbes with all the essential elements for growth. Mandel and Reese (1957), who were the pioneers of cellulase research, developed the basic medium for cellulase production.

The medium contained different cellulose preparations as the carbon source, organic and inorganic nitrogen sources and trace elements. Since then many different media compositions have been tried by researchers. Generally, in fermentation medium carbon and nitrogen sources play dominant roles as they are directly linked to the production of biomass and metabolites.

3.3.1.2.A. *Carbon sources*

Cellulase being an inducible enzyme the carbon source used plays a vital role in the productivity. A wide range of carbon sources such as CMC, starch, cellulose powder, maltose, mannitol, galactose (Goyal et al. 2014) glucose, sucrose, rhamnose, mannose, sorbitol, raffinose, trehalose (Shankar and Isaiarasu 2011), lactose, fructose (Sethi et al. 2013) arabinose, xylose, cellobiose (Premalatha et al. 2015) have been tested by researchers for cellulase production.

Sethi et al. (2013) observed highest endoglucanase production by *Pseudomonas fluorescens, Bacillus subtilis, E. coli,* and *Serratia marcescens* when glucose was used as carbon source followed by fructose for *B. subtilis, E. coli,* and *S. marcescens* and lactose for *P. fluorescens.* Whereas another strain of *Bacillus subtilis* isolated from cow dung preferred lactose for endoglucanase production followed by sucrose, mannose, starch and glucose (Bai et al. 2012). Xylose was found to be the best carbon source for CMCase production by marine *Bacillus* VITRKHB (Singh et al. 2014a). Researchers like Abou-Taleb et al. (2009), Goyal et al. (2014), Kowsalya and Gurusamy (2014), Lugani et al. (2015), and Premalatha et al. (2015) observed maximum production of endoglucanase

when the medium was supplemented with CMC as carbon source as compared to others. *Bacillus pumilus* EWBCM1 isolated by Shankar and Isaiarasu (2011) showed maximum endoglucanase production when galactose was used as the carbon source.

3.3.1.2.B. *Nitrogen sources*

Nitrogen sources form secondary energy sources for microbes and play a vital role in the cell growth and production of enzymes. Nitrogen is the constituent of amino acids, nucleic acids and coenzymes and comprises about 8-14 % of dry cell mass of bacteria. According to Sethi et al. (2013) the nitrogen and carbohydrate sources in the medium affect the extracellular production of cellulase. The nature and concentration of the source can either stimulate or down-regulate the production of enzymes.

The requirement of nitrogen differs from organism to organism. Over the years different nitrogen sources (organic or inorganic) have been used by researchers for enhancing endoglucanase production. The organic sources used include yeast extract, peptone, urea, beef extract, casein, tryptone, glycine, gelatin, alanine and malt extract. The inorganic sources used commonly are ammonium nitrate, ammonium sulphate, ammonium chloride, ammonium phosphate, ammonium molybdate, potassium nitrate, and sodium nitrate (Shankar and Isaiarasu 2011). Organic nitrogen sources like yeast extract (Acharya and Chaudhary 2012; Kowsalya and Gurusamy 2014) and peptone (Bai et al. 2012; Lugani et al. 2015) were the most favored nitrogen sources for endoglucanase production by different *Bacillus* species. Whereas a combination of both yeast extract

and peptone were required for high endoglucanase production by *Bacillus subtilis* AS3 (Deka et al. 2011), *B. amyloliquefaciens* SS35 (Singh et al. 2014b), and *Bacillus subtilis* BY-2 (Yang et al. 2014). Inorganic nitrogen sources mainly different ammonium salts like ammonium chloride (Liang et al. 2014) ammonium sulphate (Sethi et al. 2013) ammonium nitrate (Sadhu et al. 2014) were found to enhance endoglucanase production in different strains of bacteria. In a study conducted by Goyal et al. (2014), *Bacillus* sp. 313SI was found to produce maximum endoglucanase when ammonium sulphate and ammonium nitrate were used as nitrogen sources under stationary and shaking conditions, respectively.

3.3.1.2.C. *Mineral Salts*

Culture media supplies microbes with all essential elements for growth. Some microorganisms can synthesize most of their cellular components using carbon and nitrogen sources. But most of them require mineral elements for growth and metabolism in addition to carbon and nitrogen. These elements act as cofactors of essential enzymatic reactions occurring in the cell. An optimum concentration of mineral salt increases the utilization of substrate in the culture medium. The mineral salts dissociate into ions in the medium and these ions are thought to affect the catalytic activity and stability of both intracellular and extracellular enzymes (Karim et al. 2015).

Different minerals such as magnesium, phosphorous, potassium, sulphur, calcium and chlorine are essential components of media and are supplemented in the form of their salts. Sreeja et al. (2013) used variety of mineral salts to enhance endoglucanase production from two strains of

Bacillus. *B.altitudinis* showed high enzyme production when magnesium sulphate was used whereas *B. licheniformis* preferred manganous sulphate. Kowsalya and Gurusamy (2014) and Shajahan et al. (2017) reported increased endoglucanase production by *Bacillus subtilis* KG10 and *Bacillus licheniformis* NCIM 5556 respectively, when calcium chloride administered media was used. Metal ions can also have an inhibitory effect on cellulase production. Bahobil et al. (2014) reported an inhibitory effect of metal ions on cellulase production by *B. stearothermophilus* KGKSA40.

3.3.2. Optimization of cellulase production

Enzyme production in microbes is closely controlled and therefore productivity can be improved by ameliorating these controls. Cellulase yield is highly dependent on the complex interaction between various physicochemical parameters. Therefore, for overproduction of enzyme, it is necessary to investigate the significant parameters and find their optimum levels. Thus optimization studies facilitate production of large quantities of enzyme thereby reducing the production cost (Gautam et al. 2011).

Over the years different strategies have been adopted by researchers for optimization studies. The traditional OFAT approach involves changing one factor while keeping the others at constant levels. Although the method is simple and useful for screening, it has few limitations: it determines the effect of only one variable at a time and does not consider the complex interaction among them. Also, the method is time consuming, tedious and requires a number of experiments to find the

optimum levels (Zambare and Christopher 2011). In spite of these drawbacks OFAT method has been widely employed for cellulase optimization in recent years. Researchers such as Acharya and Chaudhary (2012), Kumar et al. (2012b), Lugani et al. (2015) and Hussain et al. (2017) were successful in enhancing the endoglucanase production from various strains of *Bacillus* by optimizing different environmental and nutritional factors via OFAT method. The major parameters that were considered for optimization were temperature, pH, incubation time, inoculum concentration, media volume, agitation, and nitrogen and carbon sources.

Design of Experiments or DOE is an alternative technique used to address the limitations of OFAT method. It was developed by Sir Ronald Aylmer Fisher in 1920's to study the effect of multiple variables simultaneously (Fisher 1949). DOE technique is a statistical tool that analyses the process inputs that have a significant effect on the desired output and finds the level of these inputs to achieve the desired output. It uses a systematic method to determine the optimal process parameters to attain a required product with fewer testing trails. Several DOE techniques are available to the experimental designer and the choice depends on the aim of the experiments.

Generally, DOE is carried out in four stages: planning, screening, optimization and verification. Planning the experiment is the first stage and careful planning will avoid error in the execution of the experiment. In an experiment, there may be many factors influencing the desired output. Screening, which is the second stage, reduces the number of factors by identifying the key factors essential to obtain the required

result. This reduction helps in focusing the improvement studies on the most vital factors. It also suggests the optimal range of the factors which can further be optimized to determine the best level. The main methods used for screening are general factorial design, full and fractional factorial design and Plackett-Burman design. When a search for significant variables that contribute to the final product quality is demanding, factorial design (to eliminate some independent variables that are not statistically significant) is used (Granato and de Araújo Calado 2014). A fractional or full factorial design provides complete information but requires a lot of experiments which make it impracticable when large numbers of variables are to be studied. For a rough estimate of main effects, Plackett-Burman design (PBD) is the most preferred option. The Plackett-Burman design is a two-level fractional factorial design, which allows screening and selection of most significant variables from among a large number of variables. However, it does not consider the interactions between the variables.

Next stage involves optimization of the selected vital components. The techniques used for optimization can be roughly classified into four categories: factorial design, response surface methodology (RSM), Taguchi design, and mixture experiments. Among these RSM is the most widely used method (Myers et al. 2016). This design was developed by Box and Wilson (1951). This technique enables to evaluate the optimal level of each variable, their interactions with other variables and their effect on product yield (Ali et al. 2013). For RSM purposes, there are two quadratic factorial designs: central composite design and Box-Behnken design. Box-Wilson Central Composite Design, commonly called as

central composite design (CCD), is the most efficient and popular RSM design. The last stage of DOE is validation which involves performing few experiments at the predicted optimal levels in order to confirm the results of optimization.

Plackett-Burman Design for screening and central composite design for optimization have been successfully used to enhance the production of various enzymes like cellulase (Thakkar and Saraf 2014), amylase (Cotârlet and Bahrim 2012), protease (Saxena and Singh 2014), gelatinolytic enzyme (Sai-Ut et al. 2014), xylanase (Chaturvedi et al. 2015), chitinase (Warda et al. 2016) etc. Improvement of endoglucanase production by statistical methods has been attempted by many researchers. Parkhey et al. (2016) observed a 2.8 fold increase in endoglucanase production by *Ochrobactrum haematophilum* using this method. They used the following combination of parameters: CMC (4.76 %) yeast extract (2.03 %), pH (6.3), and temperature (44.2 °C) for maximum enzyme production. Recently, endoglucanase production from many bacteria belonging to *Bacillus* spp. has been optimized by a combination of PBD and CCD (Table 3.1).

Table 3.1: Optimization of endoglucanase production by *Bacillus* spp. using Plackett – Burman and central composite design

Strains	Software used	Factors screened (g/l)	Selected components and concentration (g/l)	Fold increase	Reference
Bacillus subtilis AS3	Minitab	CMC- 10 Peptone - 5 yeast extract - 5 K_2HPO_4-1 $MgSO_4 \cdot 7H_2O$ - 0.25 $FeSO_4 \cdot 7H_2O$- 0.25 $MnCl_2 \cdot 4H_2O$- 0.5	CMC – 18 Peptone–8 Yeast extract 4.79	6	Deka et al. (2011)
Bacillus amyloliquefaciens SS35	Minitab	CMC-10 yeast extract -5 peptone - 5 K_2HPO_4 - 1 $MgSO_4 \cdot 7H_2O$ - 0.2 NaCl -1	CMC -18.05 Yeast extract - 8 peptone - 2	3	Singh et al. (2014b)
Bacillus amyloliquefaciens MBAA3	Design expert	CMC Sucrose Yeast extract Peptone K_2HPO_4 $MgSO_4$ Temperature pH	CMC -1.84 $MgSO_4$ - 0.275 pH - 8.5	6.81	Thakkar and Saraf (2014)
Bacillus licheniformis NCIM 5556	Minitab and design expert	CMC yeast extract $CaCl_2 \cdot 6H_2O$ Tween-20 pH temperature inoculum size	CMC -19.21 $CaCl_2 \cdot 6H_2O$- 25.06 mg/l Tween-20 - 2.96 ml/l temperature - 43.35 °C	3	Shajahan et al. (2017)

Response surface methodology has been extensively and effectively used to optimize selected parameters and improve product yield. Kazemi et al. (2014) used Box–Behnken design to optimize endoglucanase production by *Bacillus* sp. BCCS A3. Initially, the carbon and nitrogen sources were selected, while keeping other components at a

constant level. Later the selected sources which were CMC and tryptone were optimized along with pH. Maximum endoglucanase production was obtained using the following composition: CMC 0.75 %, tryptone 0.1 %, and pH 9.0. Shankar and Isaiarasu (2011), used galactose (1.0 g/l), malt extract (0.5 g/l) and incubation time of 72 h to obtain maximum enzyme yield from *Bacillus pumilus* EWBCM1. Singh et al. (2014a) reported maximum CMCase production by *Bacillus* VITRKHB after 24 h incubation using media containing xylose 5.0 %, beef extract 6.9 %, NaCl 1.17 % at pH 7.83 and temperature 25.84 °C. The optimized combination of variables for maximum endoglucanase production by *Enhydrobacter* sp. ACCA2 was determined as 1.5 % CMC, 1.45 % peptone, 0.044 % NaCl, 0.031 % $MgSO_4$, 0.045 % $(NH_4)_2SO_4$, and 0.15 % K_2HPO_4 (Premalatha et al. 2015).

3.4. MATERIALS AND METHODS

3.4.1. Microorganism and culture condition for endoglucanase production

The bacterium *Bacillus subtilis* MU S1 isolated from Eravikulam National Park of Munnar wildlife division was subjected to optimization studies. The seed culture was prepared by inoculating a loopful of the culture into 50 ml unoptimized medium containing following components in g/l: CMC (10.0), NaCl (6.0), $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (0.5), K_2HPO_4 (0.5), $MgSO_4$ (0.1), $CaCl_2$ (0.1), $NaNO_3$ (0.1) and Yeast extract (1.0) at pH 7.0. The culture was incubated in a shaker incubator at 37 °C. One percent of the overnight grown culture (adjusted to a McFarland standard of 1.0, approximately $3x10^8$ CFU/ml) was used as the seed for

endoglucanase production using the unoptimized medium at conditions mentioned above. After 24 h incubation, the supernatant collected following centrifugation at 10,000 g for 10 min was used to analyze enzyme activity. The endoglucanase assay was performed and activity calculated as detailed in section 2.4.5 of chapter 2.

3.4.2. Optimization of physical parameters by OFAT design

The temperature and agitation speed for endoglucanase production were optimized by OFAT method. For optimizing temperature, the unoptimized medium was inoculated with seed culture and incubated at varying temperatures (30 °C, 40 °C and 50 °C) in a shaker incubator. After 24 h the culture was centrifuged and the cell free supernatant was used as the crude enzyme for endoglucanase assay. The optimal agitation speed was determined by inoculating and incubating medium at different agitation speeds (50, 100, 150 and 200 rpm) at optimized temperature. Endoglucanase assay was performed in duplicate with crude enzyme obtained from 24 h culture. The medium components were optimized by growing the organism under the optimized physical conditions.

3.4.3. Medium optimization by statistical method

3.4.3.1. *Plackett-Burman Design for screening of significant medium components*

The most important variables that significantly influence CMCase production was selected by Plackett-Burman design using the statistical software package MINITAB (Release 16, PA, USA). A total of ten parameters were screened and each parameter was examined at its low

level (−1) and high level (+1) (Table 3.2). PBD is based on the first order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i$$

where Y is the response (CMCase activity), β_0 is the intercept of the model, β_i is the linear coefficient, and X_i is the level of the independent variable. This design does not consider the interaction among variables and a linear approach is considered to be sufficient for screening. The main effects of such a design are calculated as the difference between the average of measurements made at the high level (+1) of the factor and the average of measurements at the low level (−1) .

Table 3.2: Medium components and their variables used in Plackett-Burman design

Nutrient code	Nutrients (g/l)	Low (-1)	High (+1)
A	CMC	2	18
B	Yeast extract	0.5	10.5
C	NaCl	2	14
D	$(NH_4)_2SO_4$	0.5	2.5
E	KH_2PO_4	0.05	2.05
F	K_2HPO_4	0.05	0.45
G	$MgSO_4$	0.01	0.21
H	$CaCl_2$	0.005	0.405
I	$NaNO_3$	0.005	0.805
J	pH	5.0	7.0

The ten variables were screened in 20 experimental runs. Averages of endoglucanase activity obtained from duplicate experiments were taken as the response (Table 3.4). Regression analysis was performed to determine the factors that influence enzyme production. The

factors which were significant at or above 95 % level ($p < 0.05$) were selected and later optimized by central composite design.

3.4.3.2. *Central composite design*

After identifying the significant variables for CMCase production by PBD, response surface methodology using CCD was employed to determine the optimal levels of these variables. A 3-factor-5-level design was used and five coded levels (-α, -1, 0, +1, + α) were assigned to each factor (Table 3.3). Alpha is the extended level with the value of $(2)^{3/4}$ = 1.682. A 2^3 full-factorial CCD experimental design containing three significant medium components (CMC, yeast extract and NaCl) at five coded levels was generated using the statistical software package "Design Expert 7" (Stat Ease Inc., Minneapolis, USA). The experimental design comprised of 20 runs ($=2^k + 2k + n_0$), where 'k' is the number of independent variables and n_0 is the number of replicate runs at the center point of the variables. All experiments were carried out in duplicate and the averages of the CMCase activity were taken as the response (Table 3.6).

Table 3.3: Experimental range and levels of independent variables used for central composite design

Variables	Components	Levels				
		- α	**-1**	**0**	**+1**	**+ α**
A	CMC	2	5.24	10	14.76	18
B	Yeast extract	0.5	2.53	5.5	8.47	10.5
C	NaCl	2	4.43	8	11.57	14

The coded values of independent variables were calculated using following equation

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad i = 1, 2, 3, \ldots, k$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the value of X_i at the center point, and ΔX_i is the step change. The experimental data were analyzed using multiple regression analysis. The following second-order polynomial equation was applied to determine the relationships and interrelationships of the variables

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{23} BC + \beta_{13} AC$$

where Y is the predicted response, β_0 is the intercept, β_1, β_2, β_3 are linear coefficients, β_{11}, β_{22}, β_{33} are quadratic coefficients, and β_{12}, β_{23}, β_{13} are the interaction coefficients, A,B and C are the independent variables studied.

The results of CCD were analyzed using ANOVA (analysis of variance) test of Design Expert 7, to determine the significance of each term in the equations fitted and to estimate the goodness of fit of the model. The fitted polynomial equation was graphically represented in the form of three-dimensional surface plots. These graphs display the main and interactive effects of the selected factors on endoglucanase production.

3.4.3.3. *Validation of the model*

The statistical model for the production of endoglucanase was validated by performing experiments under five different sets of predicted conditions (Table 3.8). The combination showing highest activity was chosen as the optimized medium. To determine the effect of $MgSO_4$ and $NaNO_3$, endoglucanase production of the strain in optimized medium containing $MgSO_4$ (0.01 g/l) and $NaNO_3$ (0.005 g/l) was also determined under optimized conditions.

3.4.4. Overall optimization

To find the overall enhancement in endoglucanase production after OFAT and statistical optimization, the strain was grown in optimized media under the optimal level of physical conditions. The fold increase in enzyme activity after optimization was determined by comparing the activity obtained under unoptimized and optimized conditions.

3.4.5. Growth curve analysis

In order to determine the relationship between the time course of bacterial growth and endoglucanase production in optimized medium, a growth curve analysis was performed. *B. subtilis* MU S1 was grown in optimized medium at 40 °C. Bacterial growth and activity were measured by collecting samples after every 2 h, till the stationary phase is reached.

3.5. RESULTS

3.5.1. Production of endoglucanase under unoptimized conditions

In the study, endoglucanase was produced from *Bacillus subtilis* MU S1 by fermentation in the unoptimized medium at 37 °C in a shaker incubator. After 24 h incubation, an activity of 179.06 ± 3.2 U/ml was obtained. The production of endoglucanase from strain MU S1 was improved by optimizing a range of physical and nutritional parameters by OFAT and statistical methods respectively.

3.5.2. Optimization of physical parameters for endoglucanase production

The external factors that influence endoglucanase production: temperature and agitation speed were optimized by OFAT method. In *Bacillus subtilis* MU S1 the maximum endoglucanase production (187.08 ± 2.8 U/ml) was observed at 40 °C. The production decreased at 30 °C followed by 50 °C (Figure 3.1 A). To determine the optimum agitation speed the strain was incubated at four different agitation speeds at 40 °C. From Figure 3.1 B it is evident that the optimum agitation speed for strain MU S1 was 150 rpm (191.44 ± 3.8 U/ml). Further increase in agitation rate decreased the enzyme production.

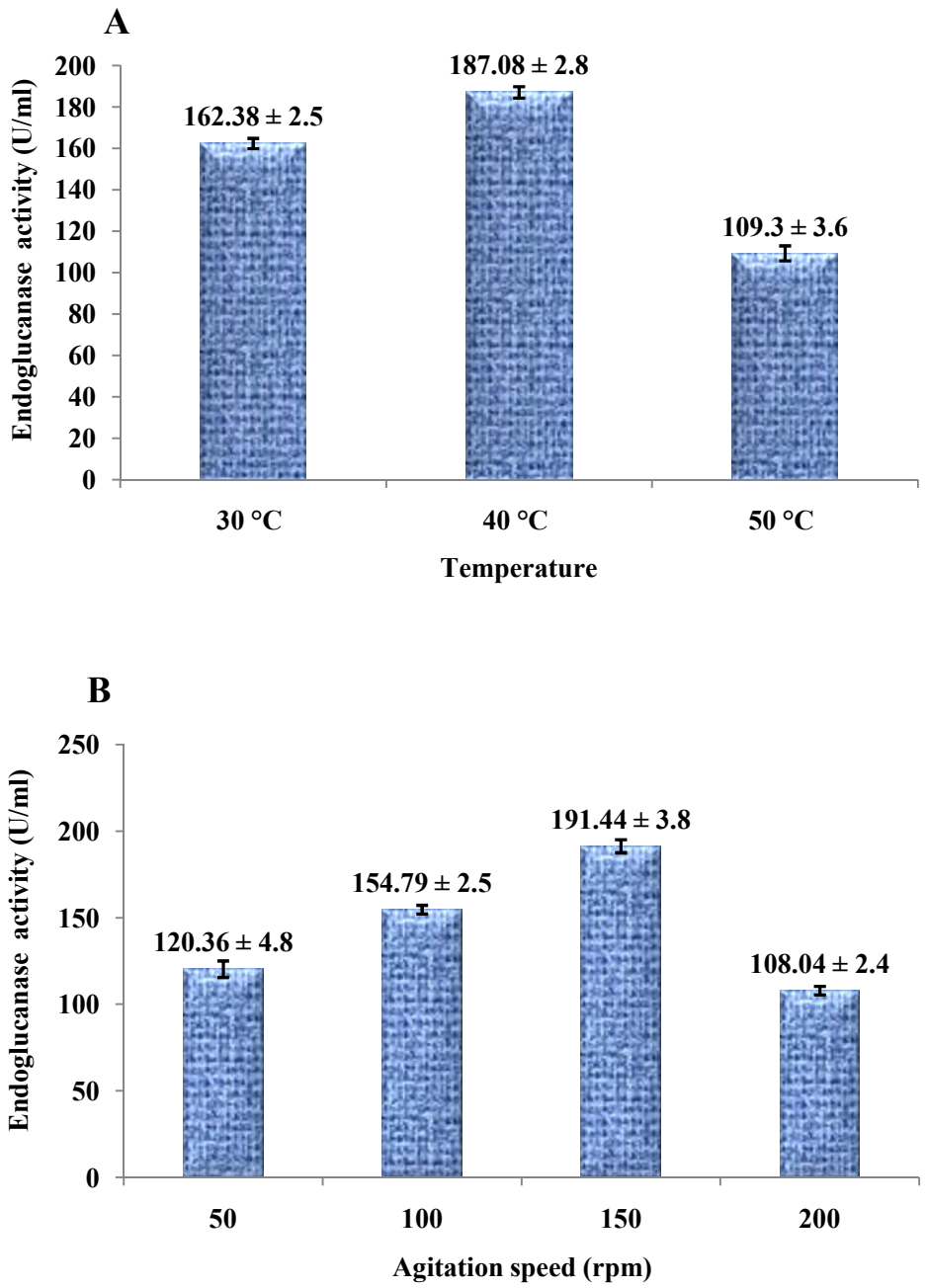


Figure 3.1: Optimization of physical parameters **(A)** temperature **(B)** agitation speed

3.5.3. Medium optimization for endoglucanase production

3.5.3.1. *Screening the significant medium components using Plackett-Burman Design*

Using PBD the most significant factors were screened from among ten components in 20 experimental runs. As indicated in Table 3.4, the CMCase activity of *Bacillus subtilis* MU S1 showed a wide variation from 8.30 to 500.80 U/ml. The important variables influencing endoglucanase production were selected based on p-value < 0.05 and confidence level $\geq$ 95 %. The components CMC, yeast extract, NaCl, pH, $MgSO_4$ and $NaNO_3$ showed $p < 0.05$. Among them CMC (p=0.00), yeast extract (p=0.00), NaCl (p=0.003) and pH (p=0.003) showed significant positive effect whereas $MgSO_4$ (p=0.006) and $NaNO_3$ (p=0.012) showed negative effect. The confidence level was approximately 100 for all the five factors. The Pareto chart (Figure 3.2) gives a pictorial representation of the significant factors. The bars that cross the reference line (t value 2.26) are statistically significant at 0.05 levels. However, the chart does not indicate the nature of effect of the factors. The overall regression coefficient for Placket-Burman design was R^2= 97.68 % with adjusted R^2 = 95.11 % and predicted R^2 = 88.56 %. The values of first order model coefficient, t- value, p-value and confidence levels of all ten variables are represented in Table 3.5.

Table 3.4: Plackett-Burman design for ten variables along with the observed results

Run	CMC	Yeast extract	NaCl	$(NH_4)_2SO_4$	KH_2PO_4	K_2HPO_4	$MgSO_4$	$CaCl_2$	$NaNO_3$	pH	Endoglucanase activity (U/ml)
1	1	-1	-1	-1	-1	1	-1	1	-1	1	190.48
2	1	-1	-1	1	1	-1	1	1	-1	-1	8.30
3	1	1	-1	1	1	1	-1	-1	-1	1	500.80
4	1	1	1	-1	-1	1	1	-1	1	1	440.32
5	-1	-1	1	-1	1	-1	1	1	1	1	10.81
6	-1	-1	-1	1	-1	1	-1	1	1	1	15.30
7	1	-1	1	-1	1	1	1	1	-1	-1	150.83
8	1	-1	1	1	1	1	-1	-1	1	1	170.69
9	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	10.53
10	1	-1	1	1	-1	-1	-1	-1	1	-1	130.40
11	-1	-1	1	1	-1	1	1	-1	-1	-1	16.34
12	-1	1	1	1	1	-1	-1	1	1	-1	320.16
13	1	1	-1	-1	1	1	-1	1	1	-1	300.64
14	1	1	1	1	-1	-1	1	1	-1	1	444.50
15	-1	1	-1	1	-1	1	1	1	1	-1	100.40
16	-1	-1	-1	-1	1	-1	1	-1	1	1	9.86
17	1	1	-1	-1	-1	-1	1	-1	1	-1	330.42
18	-1	1	1	-1	-1	-1	-1	1	-1	1	370.62
19	-1	1	1	-1	1	1	-1	-1	-1	-1	382.63
20	-1	1	-1	1	1	1	1	-1	-1	1	278.21

Table 3.5 Statistical analysis of Plackett-Burman design for ten variables

Term	Effect	Coef	SE Coef	T-Value	P-Value	Confidence level (%)
Constant		209.11	8.39	24.92	0.000	100
CMC	115.25	57.63	8.39	6.87	0.000	100
Yeast extract	275.52	137.76	8.39	16.42	0.000	100
NaCl	69.24	34.62	8.39	4.13	0.003	99.7
$(NH_4)_2SO_4$	-21.20	-10.60	8.39	-1.26	0.238	76.2
KH_2PO_4	8.36	4.18	8.39	0.50	0.630	37.0
K_2HPO_4	-9.06	-4.53	8.39	-0.54	0.603	39.7
$MgSO_4$	-60.23	-30.11	8.39	-3.59	0.006	99.4
$CaCl_2$	-35.82	-17.91	8.39	-2.13	0.062	93.8
$NaNO_3$	-52.42	-26.21	8.39	-3.12	0.012	98.8
pH	68.09	34.05	8.39	4.06	0.003	99.7

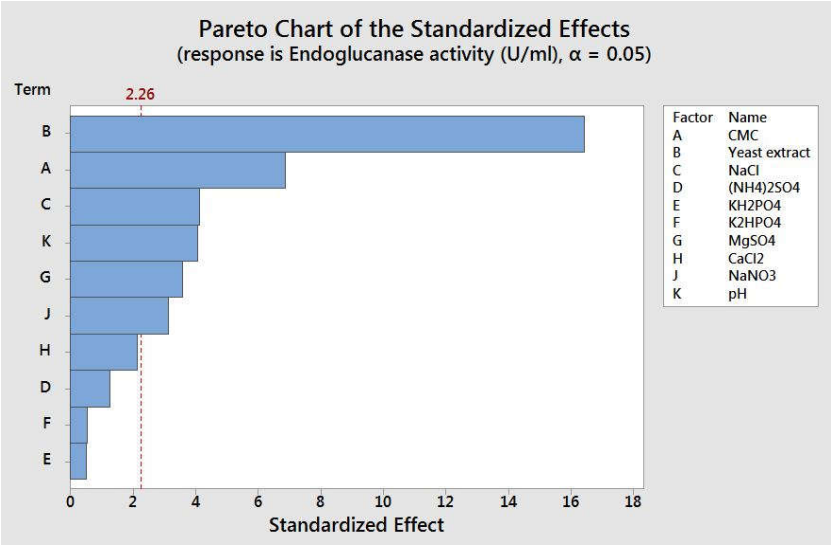


Figure 3.2: Pareto chart showing effect of medium components on endoglucanase activity

3.5.3.2. *Optimization using central composite experimental design*

Among the six factors selected in PBD, the factors which showed negative effect viz $MgSO_4$ and $NaNO_3$ were eliminated from further optimization, and CCD was carried out with CMC, yeast extract and NaCl maintaining pH at neutral, at which the strain exhibited maximum enzyme production. A full factorial CCD was performed with these 3 factors and the results were recorded in Table 3.6. The predicted endoglucanase activity was calculated using the second order polynomial equation

$$\begin{aligned}\text{Endoglucanase activity (U/ml)} = {} & + 95.48885 + 37.85381A + 34.48640B - 1.29548C \\ & + 3.43451AB - 0.78909AC + 1.96564BC \\ & - 2.07696A^2 - 5.28523B^2 - 0.54335C^2\end{aligned}$$

where A, B and C are concentrations of CMC, yeast extract and NaCl in g/l. The statistical significance of the second order polynomial equation was evaluated by the *F* test and the result of ANOVA was recorded in Table 3.7. The model was significant as indicated by "model *F*-value" of 50.29 and *p*-values less than 0.05. In the present work, linear terms A, B, C the square effect of A, B and combinations of AB and BC were found to be significant for endoglucanase activity. The insignificant "lack of fit *F*-value" (2.41) implies that the predicted results were in a good fit. The R^2 value (multiple correlation coefficient /determination coefficient) obtained was 0.9784. The predicted R^2 of 0.8747 was also in reasonable agreement with adjusted R^2 of 0.9589 which indicates that the model fits very well to the experimental data. This was confirmed by the predicted endoglucanase activity which matches well with the observed activity. The adequate precision of the present analysis was 23.672.

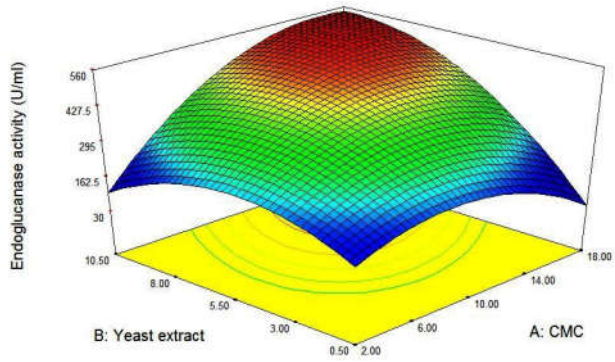
Table 3.6: Full factorial central composite design matrix with actual values of variables and the observed and predicted response.

Run	CMC	Yeast extract	NaCl	Endoglucanase activity (U/ml)	
				Observed	Predicted
1	14.76	2.53	4.43	350.26	337.44
2	10.00	5.50	2.00	492.13	486.10
3	5.24	8.47	4.43	324.28	341.22
4	5.24	2.53	11.57	268.31	257.74
5	10.00	5.50	8.00	479.33	463.25
6	14.76	8.47	11.57	506.40	514.18
7	14.76	8.47	4.43	536.32	549.79
8	14.76	2.53	11.57	232.50	218.46
9	2.00	5.50	8.00	268.03	259.19
10	18.00	5.50	8.00	396.63	401.46
11	10.00	5.50	8.00	465.26	463.25
12	5.24	8.47	11.57	343.48	359.24
13	10.00	5.50	8.00	471.32	463.25
14	5.24	2.53	4.43	328.06	323.08
15	10.00	5.50	8.00	456.53	463.25
16	10.00	5.50	14.00	399.26	401.27
17	10.00	5.50	8.00	434.32	463.25
18	10.00	5.50	8.00	472.04	463.25
19	10.00	0.50	8.00	172.19	199.02
20	10.00	10.50	8.00	494.06	463.21

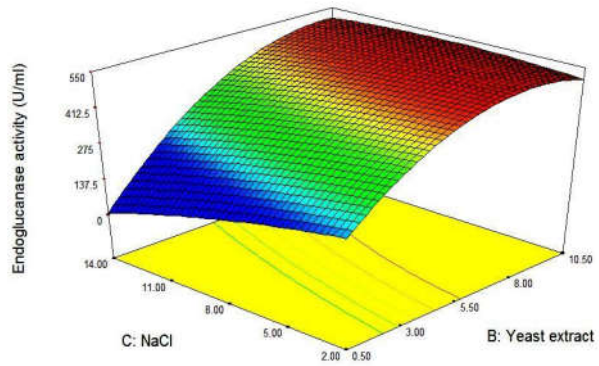
Table 3.7: Analysis of variance (ANOVA) of response surface quadratic model for the production of endoglucanase by *Bacillus subtilis* MU S1

Source	Sum of squares	df	Mean square	*F* value	*p*- value Prob > F	
Model	1.988E+005	9	22083.76	50.29	< 0.0001	significant
A-CMC	24431.30	1	24431.30	55.63	< 0.0001	
B-Yeast extract	84252.06	1	84252.06	191.85	< 0.0001	
C-NaCl	8686.03	1	8686.03	19.78	0.0012	
AB	18873.33	1	18873.33	42.98	<0.0001	
AC	1434.60	1	1434.60	3.27	0.1008	
BC	3477.36	1	3477.36	7.92	0.0184	
A^2	31829.57	1	31829.57	72.48	< 0.0001	
B^2	31449.98	1	31449.98	71.61	< 0.0001	
C^2	689.26	1	689.26	1.57	0.2388	
Residual	4391.58	10	439.16			
Lack of Fit	3104.57	5	620.91	2.41	0.1780	not significant
Pure Error	1287.02	5	257.40			
Cor Total	2.031E+005	19				

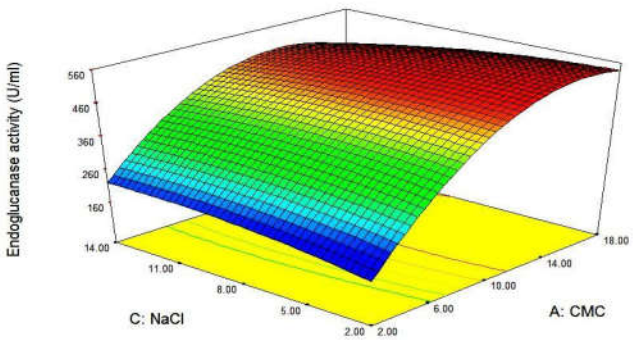
The effect of the interaction of variables on endoglucanase production was studied by three-dimensional surface curves against two independent variables while the other independent variable was maintained at its constant level. These surface plots demonstrate the individual and interactive effect of the media components and can be used to predict the optimal value of different variables. Three response surface plots were obtained by considering all the possible combinations (Figure 3.3). The 3D response surface plot shown in Figure 3.3A depicts the interaction between CMC and yeast extract. The endoglucanase activity increases with increasing concentration of both the components, however, when the concentrations reach beyond the middle value the activity tends to decline. The plot shows strong interaction between the components which was confirmed by p-value < 0.0001. Figure 3.3B shows the interaction between yeast extract and NaCl. In case of yeast extract the surface curvature is ascending indicating an increase in activity, but beyond about 8 g/l the activity decreases. For NaCl, no much surface curvature was seen but the activity tends to decrease with higher concentration. Figure 3.3C describes the interaction between CMC and NaCl. From the graph, it is evident that there is little or no interaction between the two components. High level of CMC and almost middle level of NaCl was most optimum for high endoglucanase production.



(A)



(B)



(C)

Figure 3.3: Response surface graph showing interaction effects between concentrations of selected variables **(A)** CMC and yeast extract **(B)** NaCl and yeast extract **(C)** CMC and NaCl

3.5.3.3. *Validation of the model*

In order to verify the adequacy of statistical analysis and quadratic model, a set of experiments were performed in duplicate using the combinations suggested by the software (Table 3.8). The maximum endoglucanase activity predicted by the model using the optimum concentration (13.46 g/l CMC, 8.38 g/l yeast extract and 6.31 g/l NaCl at pH 7.0) was 541.05 U/ml. This was in agreement with the experimental yield of 566.62 U/ml, which verifies the validity of the model and existence of optimal points. The presence of $MgSO_4$ and $NaNO_3$ in the optimized medium decreased the activity to 516.23 U/ml.

Table 3.8: Validation of the model

Run	CMC	Yeast extract	NaCl	Endoglucanase activity (U/ml)	
				Observed	Predicted
1	13.46	8.38	6.31	566.62	541.05
2	13.17	8.32	5.15	551.23	540.81
3	13.98	8.45	7.72	528.54	539.21
4	14.02	7.55	5.23	546.08	538.71
5	13.26	7.58	4.92	519.78	537.46

3.5.4. Overall optimization

After OFAT and statistical optimization the improvement in endoglucanase production was determined. There was an overall 3.2 fold increase in endoglucanase after optimization.

3.5.5. Growth curve analysis

The growth curve analysis showed that growth and endoglucanase activity were related to each other. Highest activity was observed in the late log phase, thereafter the activity reduced slightly (Figure 3.4).

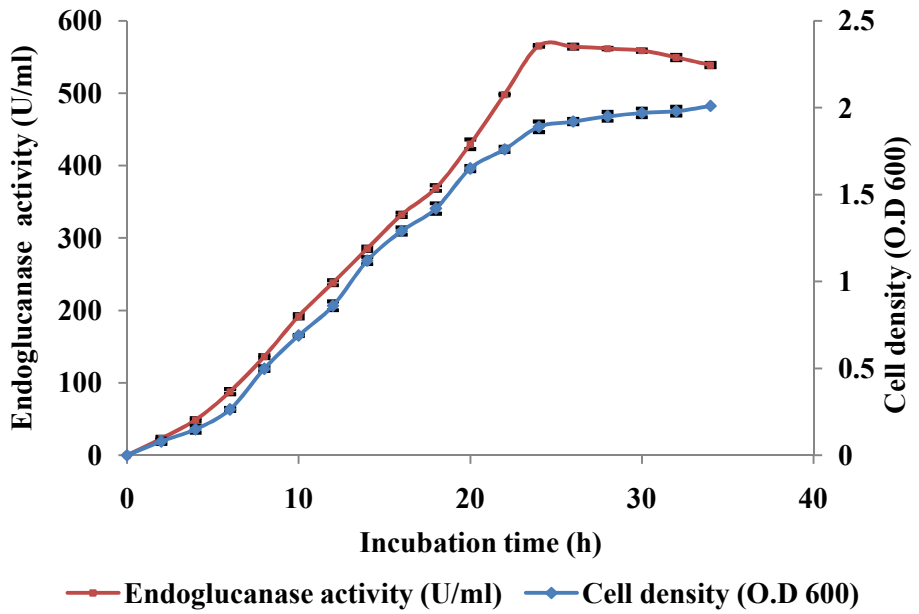


Figure 3.4: Endoglucanase production profile and growth curve of *B.subtilis* MU S1 in optimized medium

3.6. DISCUSSION

Biotechnological processes are generally highly dependent on production parameters and optimization of these parameters enhances the yield, improves reproducibility and also reduces the cost of production. There is no general medium for enzyme production by different microbial strains. Cell growth and enzyme production are greatly influenced by the

chemical composition of culture medium and environmental factors. Moreover, each strain has its own idiosyncratic physicochemical and nutritional requirements for growth and secretion of the enzyme (Reddy et al. 2008). In order to improve the enzyme production, the physical parameters and medium components were optimized. Lastly, the fold increase was monitored.

The physical parameters (temperature and agitation speed) for endoglucanase production were optimized using the traditional method of OFAT. Temperature is a critical parameter which influences extracellular enzyme production by changing the physical properties of the cell membrane. In case of *Bacillus subtilis* MU S1 the maximum endoglucanase production (187.08 ± 2.8 U/ml) was observed at 40 °C. Lower activity was observed at 30 °C and 50 °C. The studies of Immanuel et al. (2006) showed that the microorganisms belonging to *Cellulomonas*, *Bacillus,* and *Micrococcus* sp. produced maximum endoglucanase at 40 °C and neutral pH. Ray et al. (2007) also observed maximum cellulase yield at 40 °C by *Bacillus subtilis*. Agitation speed is another important culture parameter that maintains homogenous condition and disperses dissolved oxygen, thus helps in enhancing both substrate utilization and microbial activity. The optimum agitation speed for strain MU S1 was 150 rpm (191.44 ± 3.8 U/ml). Enzyme production decreased at lower and higher speeds. Cell death and enzyme inactivation due to shearing effect may be the reason for low activity at higher agitation speeds. Dash et al. (2015) tried agitation speed between 100-180 rpm and found that 150 rpm was optimum for enzyme production by *Bacillus subtilis* BI19. The

optimum temperature and agitation speed for further experiments were fixed based on OFAT results.

Media components and their interactions play vital role in extracellular enzyme production. Initially, the influence of ten variables on endoglucanase production was studied by Plackett- Burman method. Six variables viz. CMC, yeast extract, NaCl, $MgSO_4$, $NaNO_3$ and pH that significantly affect endoglucanase production were selected based on the *p* and *t* values. These factors had *p*-value < 0.05 and *t* value greater than 2.26. The sign of *t* value indicates the type of effect that is either positive or negative. Among the factors $MgSO_4$ and $NaNO_3$ were indicated to have a negative effect on endoglucanase production.

Among the selected components CMC acts as the sole carbon source. Carboxymethyl cellulose is reported as the best carbon source for endoglucanase production by many *Bacillus* spp. (Abou-Taleb et al. 2009; Goyal et al. 2014). Niranjane et al. (2007) also found that CMC was a superior carbon source for endoglucanase production when compared with cellulose. Among the nitrogen sources, yeast extract was shown to have an immense influence on enzyme production. The presence of external nitrogen source is essential in fermentation media during extracellular enzyme production for effective utilization of carbohydrates. Organic nitrogen sources are preferred for cellulase production compared to inorganic sources. In our medium yeast extract, $(NH_4)_2SO_4$ and $NaNO_3$ acted as nitrogen sources and among them, yeast extract was found to have an immense influence on enzyme production. Abou-Taleb et al. (2009) has also reported yeast extract to be the best nitrogen source. Sodium nitrate was also selected but was found to have negative influence

on endoglucanase production. The results suggest that organic nitrogen was more suitable for endoglucanase production than inorganic source. This was in accordance with the results of Ray et al. (2007) who reported that *Bacillus subtilis* preferred organic nitrogen sources. Higher enzyme production in the presence of organic nitrogen may be attributed to the vitamins and growth precursors present in it. Acharya and Chaudhary (2011), reported that *Bacillus licheniformis* WBS1 and *Bacillus* sp. WBS3 did not show any detectable CMCase activity when $(NH_4)_2SO_4$ and $NaNO_3$ was used as the sole source of nitrogen.

Sodium chloride and $MgSO_4$ were also found to influence endoglucanase production; NaCl was positively significant whereas $MgSO_4$ showed negative effect. Presence of both these components in the medium is thought to play a crucial role in initial cell growth. Researchers have demonstrated a positive effect of NaCl on endoglucanase production by different *Bacillus* species (Shankar and Isaiarasu 2011; Kowsalya and Gurusamy 2014). Whereas in case of $MgSO_4$ there are contrasting reports. Singh et al. (2014b) recorded a negative impact of $MgSO_4$ on endoglucanase activity whereas Thakkar and Saraf (2014) recorded a positive effect with confidence level > 95 %. The production of endoglucanase by *Bacillus* sp. JS14 was enhanced by the addition of NaCl and $MgSO_4$ (Singh and Kaur 2012). Another factor that was selected was initial pH of the medium. The pH of the growth medium strongly influences many enzymatic reactions by affecting the transport of chemical products and enzymes across the cell membrane (Liang et al. 2010). *Bacillus subtilis* MU S1 showed high endoglucanase production with initial pH 7.0. Optimum growth pH near neutrality has been reported

earlier for *Bacillus subtilis* KG10 (Kowsalya and Gurusamy 2014) and *Bacillus subtilis* CY5 (Ray et al. 2007). Amongst the remaining components, KH_2PO_4 had positive *t* value while $(NH_4)_2SO_4$, K_2HPO_4, $CaCl_2$ showed a negative value, but none of them was found to significantly affect endoglucanase production by *Bacillus subtilis* MU S1.

In the next stage, the components that positively affect endoglucanase production were optimized using the central composite design of RSM. The optimal levels of CMC, yeast extract and NaCl for maximum endoglucanase production were determined, maintaining the pH of the medium at neutral. From the results of ANOVA which include high *F*-value (50.29), low *p*-value (less than 0.05), non-significant lack of fit (2.41), determination coefficient close to 1 (R^2 of 0.9784), and adequate precision greater than 4 (23.672), it was confirmed that the proposed model was valid. The R^2 value of 0.9784 revealed that the model could explain 97.84 % variation in the response. Normally the R^2 value is between 0 and 1, and closer the value to 1 stronger the model and better it predicts the response. Another index for the fitness of model is Adequate Precision (AP) that measures the signal-to-noise ratio. A ratio greater than 4 is wanted for a good model. The ratio obtained for the present analysis was 23.672 which indicates an adequate signal. Thus this model can be used to navigate the design space identified by central composite design. Among the different combinations of the selected components predicted by the software, best five were selected and validated. The close similarity between the predicted and observed endoglucanse activity proves the efficiency of the model.

The direct visualization of interaction between variables and their effect on the response is possible with the help of 3D surface plots. The Figure 3.3 clearly indicates that maximum endoglucanase activity was observed at a high concentration of CMC and yeast extract and low concentrations of NaCl. Cellulases are inducible enzymes and CMC is known to have an inducing effect on cellulase production. Sadhu et al. (2014) has also reported the importance of CMC as a substrate for endoglucanase production by *Bacillus* sp. Similar observations were made by Deka et al. (2011) and Goyal et al. (2014). These facts can be justified by the report that enzymes involved in substrate degradation are generally inducible and produced only when the corresponding substrate is present in the nutrient media. One mole of CMCase production requires a large quantity of nitrogen, therefore nitrogen sources like yeast extract play a vital role in endoglucanase production. High yeast extract concentrations were found to be optimal for CMCase production by many *Bacillus* spp. (Goyal et al. 2014). The Figure 3.3B and C indicate that sodium chloride was essential but only at a lower concentration.

The cultivation of strain MU S1 in medium containing CMC, yeast extract and NaCl at pH 7.0, temperature 40 °C and agitation speed of 150 rpm significantly improved enzyme production. The optimal levels of the components were CMC 13.46 g/l, yeast extract 8.38 g/l and NaCl 6.31 g/l. The lower endoglucanase activity in the presence of $MgSO_4$ and $NaNO_3$ confirmed the output of PBD. The endoglucanase activity under unoptimized condition was 179.06 U/ml, however, after optimization, the overall activity increased by 3.2 fold as compared to the unoptimized condition. Medium components made a higher contribution towards the

improvement of enzyme production compared to physical parameters. Other researchers, Deka et al. (2013) and Singh et al. (2014b) have also opined that concentration of medium components is the foremost factor for endoglucanase production.

On growth curve analysis it was observed that the endoglucanase activity increased with increase in biomass and reached maximum in late log phase after approximately 24 h of incubation. As the strain entered stationary phase the activity started decreasing. Similar observation was made by Shajahan et al. (2017).

3.7. CONCLUSION

In the present study endoglucanase production from *Bacillus subtilis* MU S1 was enhanced by optimizing the physical parameters and medium components. Successive optimization by OFAT and statistical methods gave an overall 3.2 fold increase in endoglucanase production. A statistical approach for optimization reduced the number of parameters and experiments required for the production of enzymes thus reducing the production cost. The data reveals the basic requirement of the strain for high production of endoglucanase with minimum media components.

SREENA C. P. “IMPROVEMENT OF BIOCATALYTIC EFFICACY OF ENDOGLUCANASE FROM INDIGENOUS STRAIN, BACILLUS SUBTILIS MU S1 AND ITS APPLICATION IN SACCHARIFICATION OF AGRO-WASTES”. THESIS. DEPARTMENT OF LIFE SCIENCES, UNIVERSITY OF CALICUT, 2018.

4.1. INTRODUCTION

Lignocellulosic residues are the most abundant, inexpensive and renewable organic material on earth. Large amounts of lignocellulosic biomasses are generated world wide through forestry, agricultural and agro-industrial practices. Unfortunately, most of these wastes are dumped or burnt causing a pollution problem. The chemical composition of lignocellulosic wastes, consisting of three groups of polymers, namely cellulose, hemicelluloses and lignin make them an attractive resource for various biotechnological applications (Mussatto and Teixeira 2010). These wastes can be exploited for the production of value-added products like ethanol, food additives, organic acids, enzymes, and others. The bioconversion of agro-wastes is a remedy for solid waste disposal and also helps in the development of beneficial products from inexpensive sources.

Endoglucanase or endo-β- 1, 4 - glucanase is one of the major hydrolytic enzymes and has great significance in modern biotechnology. It is the first and the major component of cellulolytic enzyme system and thus plays a crucial role in the degradation of cellulosic biomass. Although the enzyme has been used in industries like paper and pulp, food and feed, detergent and textile, medical/ pharmaceutical, brewing and agriculture, its utility in biofuel industries have brought it to the limelight (Bhat 2000). But the cost of the enzyme is the major limitation in its widespread use. Thus the low-cost production of these enzymes has become the topic of current research.

Extracellular enzyme production is greatly influenced by media components and environmental factors. Among media components, carbon source plays a major role. The cost of the carbon source is a vital factor that governs the economics of endoglucanase production. It contributes to 40-60 % of production cost and thus use of cheaply available lignocellulosic residues can result in considerable reduction in enzyme production cost (Salihu et al. 2015) . In India, some of the most commonly used agricultural wastes for endoglucanase production by bacteria are sugarcane (*Saccharum officinarum*) bagasse, rice (*Oryza sativa*) straw, wheat (*Triticum aestivum*) straw, wheat bran, rice bran, maize (*Zea mays*) bran and groundnut (*Arachis hypogaea*) soy (*Glycine max*), and coconut (*Cocos nucifera*) cake (Sethi et al. 2013; Gaur and Tiwari 2015). But there is still an increasing need for exploring novel and diverse lignocellulosic wastes for production of a cost-effective media.

India, being an agricultural state, an ample amount of lignocellulosic waste is generated every year. These agro-wastes could be judiciously utilized for production of various value-added products. Pepper (*Piper nigrum*), arecanut (*Areca catechu*), tamarind (*Tamarindus indica*), banana (*Musa*) and jackfruit (*Artocarpus heterophyllus*) are some of the waste generating crops which have not much been studied for cellulase production by bacteria. Arecanut is a plantation crop, banana and jackfruit are fruit crops and pepper and tamarind are spice crops. Banana occupies the largest area of cultivation in India followed by arecanut, jackfruit, pepper and tamarind. Some of the major states in India involved in cultivation of these crops are Kerala, Tamil Nadu, Karnataka, Maharashtra, Madhya Pradesh, Andhra Pradesh, Tripura, Assam, West

Bengal, Orissa and Bihar. According to the agricultural statistics of the year 2015-2016 presented by Department of Economics and Statistics of Kerala State, arecanut occupied the largest area (99,126 ha) followed by jackfruit, pepper, banana and tamarind. These wastes can act as efficient substrates for bacteria and fungi which produce extracellular cellulases.

Presently, fermentation technology is regarded as the most potent tool for economical environment-friendly bioconversion of lignocellulosic wastes into valuable products. Utilization of lignocellulosic residue for enzyme production not only reduces the production cost but also leads to waste management (Mussatto and Teixeira 2010). However, the process of fermentation is influenced by various factors which can be carefully altered to increase the product yield. Traditionally most of the industrially important enzymes have been produced by SmF. This technique has the advantage of easy handling and better monitoring of fermentation conditions compared to SSF. Moreover, bacteria are known to prefer SmF over SSF (Subramaniyam and Vimala 2012). Production of endoglucanase using agro-waste has been carried out by both submerged and solid state fermentation.

4.2. OBJECTIVES

The prime focus of this chapter was to investigate the potential of few locally available agro-wastes as a substrate for endoglucanase production by *B. subtilis* MU S1. Later the agro-waste medium showing the highest endoglucanase production was tested for production of other cellulases and statistically optimized.

4.3. REVIEW OF LITERATURE

Enzyme production is greatly affected by the composition of fermentation media. Optimization studies can bring down the cost to some extent. But the high cost of media components mainly the carbon source has prompted the researchers to look for more economical sources for enzyme production. Use of lignocellulosic biomass as raw material for enzyme production can cause considerable cost reduction. Plant biomasses contain about 50 % of cellulose and hence are attractive sources for cellulase production. But the recalcitrant nature of lignocelluloses is a major obstacle in commercial utilization of agro-wastes. In nature, cellulose is closely interlinked with hemicelluloses, lignin and pectin. Thus pretreatment is an essential step to expose cellulose for microbial degradation and high level of enzyme production. This is achieved by breaking the lignin covering and distorting the organizational structure of lignocellulosic substrates (Salihu et al. 2015).

An efficient pretreatment must accomplish the following: preserve the hemicellulose fraction, yield maximum fermentable sugars, limit degradation of carbohydrate, minimize the formation of inhibitory byproducts and reduce energy input. It should also make the process economical and cost effective (Anwar et al. 2014). Depending on the application and type of substrate, pretreatment methods can be classified into: physical, physico-chemical, chemical (alkali, acid, oxidizing agents and organic solvents) and biological or a combination of these. Each pretreatment method has its own pros and cons and often a combination of pretreatments is preferred. The best method of pretreatment depends entirely on the type of biomass (Kumar et al. 2009b). Cellulase production

using agro-wastes have been carried out using both SSF and SmF. Large-scale commercial production of the enzyme is still using SmF technology.

Researchers over the world have used a variety of agricultural residues for endoglucanase production by microorganisms. Among them, there are numerous reports on the production of cellulases by fungi using agro-wastes (Sharada et al. 2013). However, bacteria have been scarcely exploited for endoglucanase production using agro-wastes. Among bacteria *Bacillus* species are known to be promising candidates in bioconversion studies. Most of the studies on bacterial endoglucanase production from agro-wastes are carried out under submerged fermentation using size reduced material. Size reduction considerably increases the surface area leading to greater enzyme production.

Sugarcane bagasse, wheat bran, rice bran, maize bran, wheat straw, rice straw, rice husk, and sawdust (Acharya and Chaudhary 2012; Liang et al. 2014; Padilha et al. 2015) are some of the most commonly used agro-wastes for endoglucanase production. Among these, sugarcane is widely exploited agro-waste. Kumaran et al. (2015) and Padilha et al. (2015) used sugarcane as a substrate for endoglucanase production by *B. licheniformis* MTCC 429 and *Bacillus* sp. C1AC5507 respectively using submerged fermentation. They employed different pretreatment methods to trim down the intricate nature of sugarcane bagasse. Kumaran et al. (2015) applied physical and chemical pretreatment methods. In this size reduction of the substrate was followed by chemical treatment with 2 % NaOH, whereas Padilha et al. (2015) used only size reduction method. Waghmare et al. (2014) utilized powdered agro-wastes such as sugarcane bagasse, sugarcane barbojo, sorghum (*Sorghum bicolor*) husks,

grass (*Poaceae*) powder, corn (*Zea mays*) straw and paddy straw for endoglucanase production and found that grass powder and sugarcane barbojo were best substrates. The studies of Gaur and Tiwari (2015) revealed sugarcane bagasse as most suitable for enzyme production by *Bacillus vallismortis* RG-07 from among other substrates like rice bran, rice husk, wheat bran, and maize brans. Siripornadulsil et al. (2014) also observed high cellulase activity when bagasse was used as substrate compared to rice straw and rice husk.

Agricultural residues like wheat bran, wheat straw, sawdust (Nizamudeen and Bajaj 2009), rice husk (Kumar et al. 2009a), molasses (Shabeb et al. 2010), and corn husks (Nema et al. 2015) have been used as a substrate for endoglucanase production by different *Bacillus* species. Mishra and Pandey Lata (2007) evaluated the potential of five bacterial and four fungal strains for enzyme production using paddy straw as a carbon source in submerged fermentation. Chemically pretreated rice and wheat straw were used for endoglucanase production by *Bacillus licheniformis* MVS1 and *Bacillus* sp. MVS3 by Acharya and Chaudhary (2012). Sethi et al. (2013) studied the effect of groundnut cake, coconut cake, soy cake, and wheat bran on endoglucanase production by *Pseudomonas fluorescens*, *Bacillus subtilis*, *E. coli*, and *Serratia marcescens* and reported that coconut cake supported maximum enzyme production by all strains of bacteria. Meng et al. (2014) used agro-wastes like corn stover, wheat bran, and rice straw along with CMC, soluble starch and glucose for cellulase production from *B. subtilis* BY-3. Corn stover was found to be most suitable for high enzyme production.

Other than the commonly used agro-wastes there is always need to explore novel and cheap sources to test their suitability for enzyme production. In 1999, Krishna utilized banana fruit stalk waste, rice straw, rice bran and wheat bran to produce endoglucanase by *Bacillus subtilis* CBTK 106 (Krishna 1999). He observed about 4-10 fold higher endoglucanase production from banana fruit stalk waste compared to the other traditional cellulosic substrates (rice straw, rice bran and wheat bran) used. Dabhi et al. (2014) also opined that banana waste could be utilized as an alternative substrate for low – cost enzyme production. Arooj et al. (2017) used banana peduncle for cellulase production from *Bacillus subtilis* K-18. Samuel et al. (2010) used alkali pretreated saw dust and coir waste for endoglucanase production by four bacterial spp. (*Bacillus* sp, two *Pseudomonas* spp. and *Proteus* sp.) and two fungal spp. (*Aspergillus niger* and *Aspergillus fumigatus*) under solid state fermentation. They observed that coir waste was best carbon source compared to sawdust as it gave better enzyme yield. Shivanand et al. (2013) used agro-wastes like groundnut shells and sunflower (*Helianthus*) seeds in addition to wheat bran, rice bran, corn cobs and sugarcane bagasse for endoglucanase production by *Halomonas* sp. strain PS47.

Abo-State et al. (2013) isolated bacteria from seven agricultural wastes and also tested their suitability as a substrate for cellulase production. They used new wastes, potato (*Solanum tuberosum*) peel and banana peel along with wheat bran, rice straw, wheat straw, corn cob, sugarcane bagasse. Alshelmani et al. (2014) used physically treated palm (*Arecaceae*) kernel cake as an effective substrate for endoglucanase production by four strains of bacteria. The production of cellulolytic and

hemicellulolytic enzymes by *Bacillus licheniformis* using different agro-waste based media was studied by Seo et al. (2014). They used powdered wheat bran, distillers dried grain with soluble, copra meal, and palm kernel meal, among which copra meal gave the highest yield of the enzyme. Hemavathy E et al. (2016) reported the use of alkali treated substrates, tamarind seed powder and coconut shell powder as a cellulosic carbon source. Recently, Roopa et al. (2017) studied the effect of alkali pretreatment of three substrates namely hay, fibre waste of palmyra palm (*Borassus flabellifer*), and banana bracts on cellulase production by *Bacillus subtilis*. Fiber waste of palmyra palm showed highest yield with or without treatment, followed pretreated banana bracts. But the pretreatment of hay was found to reduce enzyme activity.

4.3.1. Optimization of endoglucanase production using agro-waste as substrate

Production of industrially significant enzymes is greatly influenced by physicochemical characteristics of the medium. Optimization of fermentation condition enhances the product yield. Conventional methods are time-consuming and do not predict the combinatorial effect of different parameters, hence statistical methods are being used extensively.

The traditional method of 'one factor at a time' was adopted by Acharya and Chaudhary (2012) to obtain increased enzyme production by *Bacillus licheniformis* MVS1 and *Bacillus* sp.MVS3. Optimal levels of different environmental and nutritional factors such as pH, temperature, incubation time and nitrogen source were determined when wheat and

rice straw were used as sole carbon source. In another study conducted by Goyal et al. (2014), the enzyme production by *Bacillus* sp. 313SI was optimized using alkali assisted acidic pretreated rice straw as substrate. They optimized the effect of fermentation variables like pH, incubation temperature, incubation time, inoculum concentration, and carbon and nitrogen sources on enzyme production under stationary and shaking condition. The optimum conditions for maximum enzyme production under stationary conditions were 1% pretreated rice straw, 1% inoculum, ammonium sulphate as a nitrogen source, pH 8.0, temperature 35 °C and incubation time of 60 h. While under shaking condition the optimal levels varied (1 % pretreated substrate, 0.4 % inoculum, ammonium nitrate as nitrogen source, pH 8.0, temperature 30 °C and incubation time of 48 h). Carboxyl methyl cellulose was found to be the most optimal carbon source under both cultural conditions.

Over the years statistical methods of optimization have been widely used by researchers. Plackett-Burman design for screening and central composite design for optimization of selected factors was used by Ali et al. (2013) to increase cellulase produced by *Cellulomonas fimi* NCIM-5015 using alkali pretreated tapioca (*Manihot esculenta*) stem powder as the sole carbon source. The optimal levels of selected factors were yeast extract 2.5 g/l, peptone 0.957 g/l, KH_2PO_4 3.09 g/l, and $CaCl_2.2H_2O$ 0.409 g/l. Cellulase production by *Bacillus amyloliquefaciens* UNPDV-22 (Zambare and Christopher 2011) was optimized using the central composite design of response surface methodology. A 70 % increase in enzyme production was observed by optimizing three medium components: wheat bran (1.03 %), soybean

meal (2.43 %) and malt dextrin (2.95 %). Selvam et al. (2014) studied the effect of the combination of coffee (*Coffea*) pulp waste and pineapple (*Ananas comosus*) waste residues for endoglucanase production by *Acinetobacter* sp. TSK-MASC. Incubation time, pH, concentrations of coffee pulp waste and pineapple waste residues were optimized. They obtained higher enzyme production after 60 h of incubation with 3.0 g /l of each waste at pH 7.0. Dave et al. (2015) obtained enhanced endoglucanase production by *Bacillus licheniformis* MTCC 429 using untreated jatropha (*Jatropha curcas*) deoiled seed cake after optimization. Initial pH (pH 9.0), moisture ratio (1:1) and incubation time (72 h) were found to be ideal parameters for optimum enzyme production. Padilha et al. (2015) employed RSM to optimize temperature and pH for endoglucanase production by *Bacillus* sp. C1AC5507 using sugarcane bagasse as substrate. The optimum production was obtained at 70 °C and pH 7.0, respectively.

4.4. MATERIALS AND METHODS

4.4.1. Screening of media for endoglucanase production

Bacillus subtilis MU S1 was grown in three different media at neutral pH to evaluate the influence of media composition on endoglucanase production. The activity obtained was compared with that from original media used previously in this work. The media were formulated by changing few micro-nutrients and trace elements (Table 4.1).

Table 4.1: Composition of different media

Original medium	M 1	M 2	M 3	g/l
CMC	CMC	CMC	CMC	10
Yeast extract	Yeast extract	Yeast extract	Yeast extract	1
NaCl	NaCl	NaCl	NaCl	6
$(NH_4)_2SO_4$	$(NH_4)_2SO_4$	$(NH_4)_2SO_4$	$(NH_4)_2SO_4$	1
KH_2PO_4	KH_2PO_4	KH_2PO_4	KH_2PO_4	0.5
K_2HPO_4	Na_2HPO_4	Na_2HPO_4	K_2HPO_4	0.5
$MgSO_4$	$MnSO_4$	$FeSO_4$	$MnSO_4$	0.1
$NaNO_3$	KCl	KCl	$FeSO_4$	0.1
$CaCl_2$	$CaCO_3$	$MgSO_4$	$CaCO_3$	0.1

4.4.2. Collection and pre-treatment of agro-wastes

Agro-wastes used in this study viz pepper waste (PW), banana peduncle (BP), arecanut husk (AH), tamarind husk (TH) and jackfruit outer rind (JOR) were collected from local farms in Calicut, Kerala. In Kerala, these wastes are generated in huge amount as part of agricultural practices. These wastes were cut into small pieces, dried in hot air oven at 80 °C, milled and sieved through 1 mm mesh. The powdered wastes were pretreated by steam explosion using an autoclave. One gram of waste in 100 ml flask was subjected to sudden steam depressurization by fully opening the steam exhaust valve after autoclaving at 15 psi, and for 1 h (Sharma et al. 2007). Steam explosion disrupts cell wall, causes delignification, degradation of hemicelluloses and exposes cellulose to enzymes. This pretreated waste was used as carbon source in culture media.

4.4.3. Screening of agro-wastes as alternative carbon source

The fermentation media used for the production of endoglucanase contained the following components in g/l: CMC (10.0), NaCl (6.0), $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (0.5), Na_2HPO_4 (0.5), $MnSO_4$ (0.1), $CaCO_3$ (0.1), KCl (0.1) and Yeast extract (1.0). The pH of the medium was adjusted to 7.0. The suitability of agro-waste as alternative substrate was tested by replacing CMC in the medium with 1% of different agro-wastes. The sterilized media were inoculated with 1% of overnight grown inoculum (adjusted to a McFarland standard of 1.0, approximately $3x10^8$ CFU/ml) and incubated at 40 °C for 4 days in an orbital shaker at 150 rpm. Inoculated CMC broth was kept as the control. After every 24 h fermentation extracts were collected, centrifuged at 10,000 g for 10 min and the clear supernatants were used as the crude enzyme for endoglucanase assay. The assay was performed in duplicate as mentioned in section 2.4.5 of chapter 2.

4.4.4. Hydrolytic enzyme production using arecanut husk medium

The potential of arecanut husk medium for the production of various hydrolytic enzymes (FPase, avicelase, β-glucosidase and xylanase) was examined after 48 h incubation.

4.4.5. Statistical optimization of endoglucanase production from arecanut husk medium

The medium components required for effective utilization of arecanut husk by *Bacillus subtilis* MU S1 was statistically optimized. Initially, nine variables were screened by Plackett-Burman Design and

then the selected variables were optimized using central composite design.

4.4.5.1. *Plackett-Burman Design for screening of significant medium components*

PBD using the software package MINITAB (Release 16, PA, USA) was employed to screen the important variables that influence endoglucanase production. Nine parameters were screened and each parameter was examined at its low level (−1) and high level (+1) (Table 4.2).

Table 4.2: Variables screened using Plackett- Burman design

Nutrient code	Nutrients (g/l)	Low (-1)	High (+1)
A	Yeast extract	0.5	10.5
B	NaCl	2	14
C	$(NH_4)_2SO_4$	0.5	2.5
D	KH_2PO_4	0.05	0.45
E	Na_2HPO_4	0.05	4.05
F	$MnSO_4$	0.05	0.25
G	KCl	0.05	0.45
H	$CaCO_3$	0.05	0.85
I	Inoculum size	1 %	5 %

Nine variables were screened in 20 experimental runs. All experiments were carried out in duplicates and the averages of endoglucanase activity were used as the response (Table 4.5). The concentration of arecanut husk which served as the sole carbon source in all the media was kept constant at 1 %. After regression analysis, the

factors that were significant at or above 95 % level ($p < 0.05$) were selected and their concentrations were optimized by CCD.

4.4.5.2. *Central composite design*

The optimal levels of the variables selected by PBD were determined by CCD. The experiment was designed and analyzed using the statistical software package "Design Expert 7" (Stat Ease Inc., Minneapolis, USA). A 3-factor-5-level design was generated and the effect of each factor on endoglucanase production was analyzed at five levels (Table 4.3).

Table 4.3: Experimental range and levels of independent variables used for central composite design

Variables	Components	Levels				
		- α	-1	0	+1	+ α
A	Yeast extract (g/l)	0.5	2.53	5.5	8.47	10.5
B	Na_2HPO_4(g/l)	0.05	0.86	2.05	3.24	4.05
C	Inoculum size (%)	1.0	1.81	3.00	4.19	5.0

The experimental design comprised of 20 runs with varying composition of selected components (yeast extract, Na_2HPO_4 and inoculums size). All experiments were carried out in duplicates and the averages of the endoglucanase activity were taken as the response (Table 4.7). The data obtained from 20 runs were fitted into the second-order polynomial equation to determine the relationships and interrelationships of the variables.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{23} BC + \beta_{13} AC$$

where Y is the predicted response (endoglucanase activity U/ml), β_0 is the intercept, β_1, β_2, β_3 are linear coefficients, β_{11}, β_{22}, β_{33} are quadratic coefficients, and β_{12}, β_{23}, β_{13} are the interaction coefficients, A, B and C are the independent variables studied.

The results obtained from central composite design were examined using ANOVA. The fitted polynomial equation was graphically represented as three-dimensional surface plots which display the main and interactive effects of the selected factors on endoglucanase production.

4.4.5.3. *Validation of the model*

The adequacy of the obtained model was determined by performing experiments under a predicted set of conditions. Five different media with varying concentration of selected components were used for enzyme production (Table 4.9). The medium showing highest activity was chosen as the optimized medium.

4.4.6. Fold increase

In order to predict the fold increase in the endoglucanase production after statistical optimization, the activity obtained by growing the strain in unoptimized arecanut husk medium was compared with that from the optimized medium.

4.5. RESULTS

4.5.1. Screening of media for endoglucanase production

The enzyme activities obtained in the three newly formulated media were compared with original medium. M1 showed highest endoglucanase activity 298.86 U/ml followed by M 2. Whereas, in M 3 the production was lower than original medium (Figure 4.1). M 1 was used for all further studies.

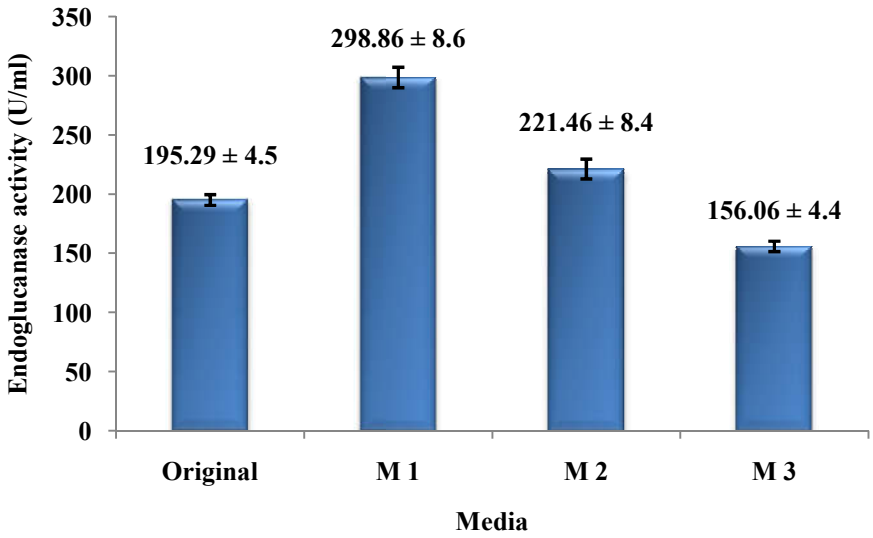


Figure 4.1: Comparison of media for endoglucanase production

4.5.2. Screening of agro-wastes as alternative carbon source

As evidenced by the data presented in the Figure 4.2, *Bacillus subtilis* MU S1 could utilize all the agricultural residues used as carbon sources but at different degrees. Among the agro-wastes, highest endoglucanase activity of 328.8 ± 5.2 U/ml was observed in AH medium after 48 h incubation, followed by TH (214.19 ± 5.2 U/ml), PW (187.34 ±

4.4 U/ml), BP (174.7 ± 3.4 U/ml) and JOR (164.91 ± 3.4 U/ml). In CMC medium the highest activity (304.86 ± 5.1 U/ml) was seen after 24 h of incubation (Table 4.4).

Table 4.4: Screening of agro-wastes for endoglucanase production

Carbon source in media	Incubation time (h)			
	24	48	72	96
CMC	**304.86 ± 5.1**	244.2 ± 2.7	229.99 ± 3.4	179.44 ± 4.7
Pepper waste	182.6 ± 6.7	**187.34 ± 4.4**	175.02 ± 2.4	144.06 ± 3.6
Banana peduncle	155.43 ± 2.9	**174.7 ± 3.4**	150.38 ± 4.2	133.95 ± 4.1
Arecanut husk	222.09 ± 4.1	**328.8 ± 5.2**	224.3 ± 2.9	164.28 ± 3.9
Tamarind husk	61.92 ± 4.7	**214.19 ± 5.2**	204.08 ± 6.3	146.58 ± 3.7
Jackfruit outer rind	75.19 ± 3.2	**164.91 ± 3.4**	102.99 ± 5.5	86.56 ± 4.1

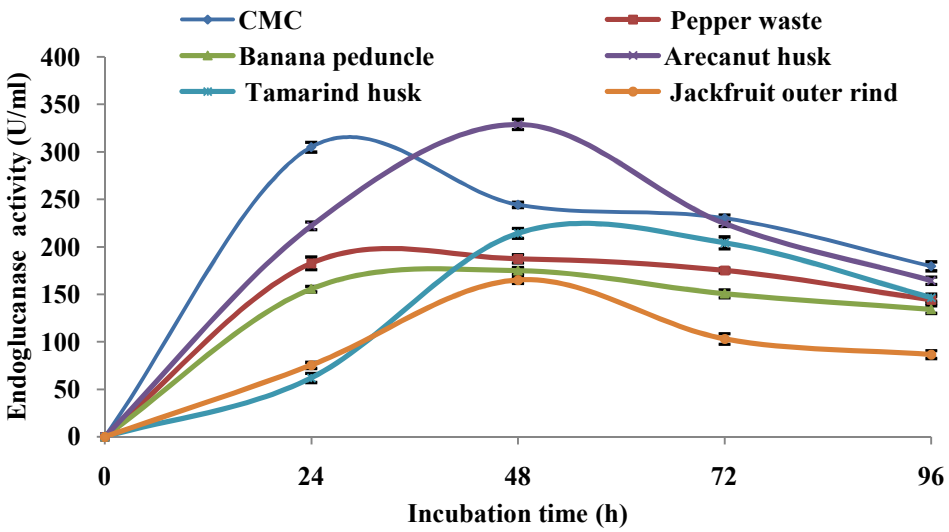


Figure 4.2: Screening of agro-wastes for endoglucanase production

4.5.3. Hydrolytic enzyme production using arecanut husk medium

It was observed that the strain produced all the hydrolytic enzymes using AH as the sole carbon source. Xylanase production was highest with the activity of 1240.91 ± 4.0 U/ml. The FPase, β-glucosidase and avicelase activities were 151.01 ± 5.7, 122.89 ± 5.2 and 79.61 ± 3.1 U/ml respectively.

4.5.4. Statistical optimization of endoglucanase production from arecanut husk medium

4.5.4.1. *Screening the significant medium components using Plackett-Burman Design*

The different variables that significantly influence endoglucanase production by *Bacillus subtilis* MU S1 when arecanut husk was used as the sole carbon source were screened from among nine components in 20 experimental runs. The endoglucanase activity in the 20 media varied from 43.92 to 692.49 U/ml (Table 4.5).

Table 4.5: Plackett-Burman design for nine variables along with the observed results

Run	Yeast extract	NaCl	$(NH_4)_2SO_4$	KH_2PO_4	Na_2HPO_4	$MnSO_4$	KCl	$CaCO_3$	Inoculum size	Endoglucanase activity (U/ml)
1	-1	-1	-1	-1	-1	-1	-1	-1	-1	43.92
2	1	-1	1	1	-1	-1	-1	-1	1	682.38
3	1	-1	1	1	1	1	-1	-1	1	689.89
4	-1	-1	-1	-1	1	-1	1	-1	1	185.95
5	1	1	1	-1	-1	1	1	-1	1	672.27
6	-1	-1	-1	1	-1	1	-1	1	1	109.46
7	1	1	-1	1	1	-1	-1	-1	-1	659.63
8	-1	-1	1	1	-1	1	1	-1	-1	44.86
9	1	-1	-1	1	1	-1	1	1	-1	665.95
10	1	-1	-1	-1	-1	1	-1	1	-1	620.09
11	1	1	-1	-1	1	1	-1	1	1	692.49
12	1	-1	1	-1	1	1	1	1	-1	680.48
13	-1	-1	1	-1	1	-1	1	1	1	189.42
14	1	1	-1	-1	-1	-1	1	-1	1	632.46
15	-1	1	-1	1	-1	1	1	1	1	87.82
16	-1	1	1	-1	-1	-1	-1	1	-1	59.39
17	1	1	1	1	-1	-1	1	1	-1	559.34
18	-1	1	-1	1	1	1	1	-1	-1	121.09
19	-1	1	1	1	1	-1	-1	1	1	156.37
20	-1	1	1	-1	1	1	-1	-1	-1	100.46

The important variables influencing endoglucanase production were selected based on p-value < 0.05 and confidence level $\geq$ 95 %. The components yeast extract, Na_2HPO_4 and inoculum size with p=0.00 and 100 % confidence level were shown to have a positive effect on endoglucanase production. As observed from Pareto chart (Figure 4.3) the t values for all three factors were higher than the limit (2.23) which indicates their significance. The overall regression coefficient for Placket-

Burman design was R^2= 99.68 % with adjusted R^2 = 99.40 % and predicted R^2 = 98.74 %. The values of the first-order model coefficient, *t*-value, *p*-value and confidence levels of all nine variables are represented in Table 4.6.

Table 4.6: Statistical analysis of Plackett-Burman design for nine variables

Term	Effect	Coef	SE Coef	T-Value	P-Value	Confidence level (%)
Constant		382.69	4.91	77.94	0.000	100
Yeast extract	545.62	272.81	4.91	55.56	0.000	100
NaCl	-17.11	-8.55	4.91	-1.74	0.112	88.8
$(NH_4)_2SO_4$	1.60	0.80	4.91	0.16	0.874	12.6
KH_2PO_4	-10.01	-5.01	4.91	-1.02	0.332	66.8
Na_2HPO_4	62.97	31.49	4.91	6.41	0.000	100
$MnSO_4$	-1.59	-0.79	4.91	-0.16	0.875	12.5
KCl	2.56	1.28	4.91	0.26	0.800	20.0
$CaCO_3$	-1.21	-0.60	4.91	-0.12	0.904	9.6
Inoculum size	54.33	27.17	4.91	5.53	0.000	100

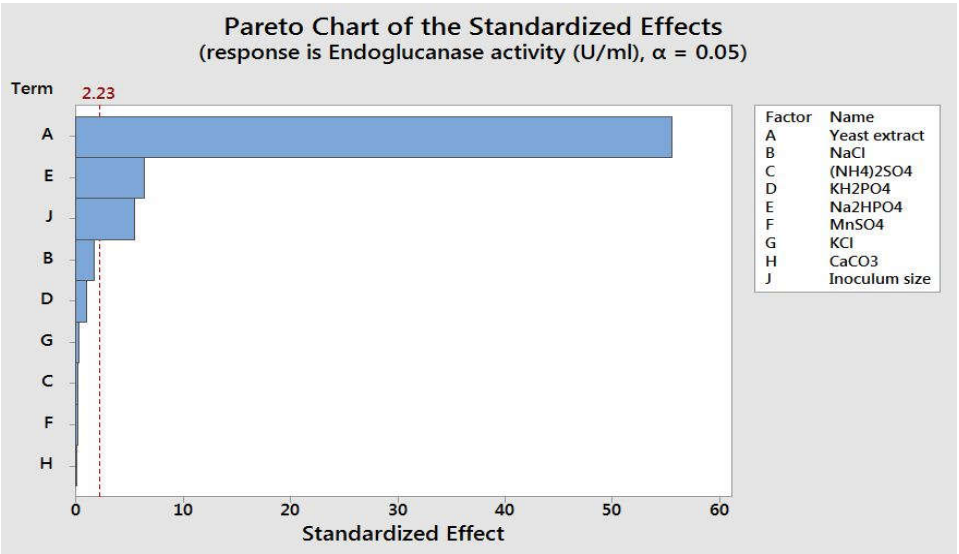


Figure 4.3: Pareto chart showing effect of medium components on endoglucanase activity

4.5.4.2. *Optimization by central composite experimental design*

The concentrations of the variables selected by PBD, namely yeast extract, Na_2HPO_4 and inoculum size were optimized using CCD. A full factorial CCD was performed with these three factors and the results were recorded in Table 4.7. The predicted endoglucanase activity was calculated using the second order polynomial equation

$$\text{Endoglucanase activity (U/ml)} = +48.22397 + 139.94286A - 129.59344B + 83.29047C - 9.73509AB - 6.87626AC + 25.86331BC - 5.03874A^2 + 13.15413B^2 - 19.32212C^2$$

where A, B and C are concentrations of yeast extract, Na_2HPO_4 in g/l and inoculum size in percentage.

The statistical significance of the second order polynomial equation was evaluated by the *F* test and the result of ANOVA was recorded in the Table 4.8. The model and model terms were significant as indicated by "model *F*-value" of 32.65 and *p*- values less than 0.05. In this study, linear terms A, B the square effect of A, C and combinations of AB and BC were found to be significant for endoglucanase activity. The insignificant "lack of fit *F*-value" (2.96) implies that the predicted results were in a good fit. The R^2 value obtained was 0.9671. The predicted R^2 of 0.7984 was also in reasonable agreement with adjusted R^2 of 0.9375 which indicates that the model fits very well to the experimental data. This was confirmed by the predicted endoglucanase activities which match well with the observed activity. The adequate precision for the present analysis was 24.236 which was much higher than the desirable value.

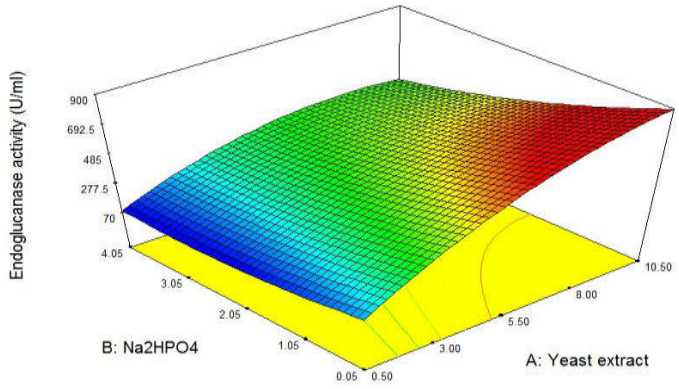
Table 4.7: Full factorial central composite design matrix with actual values of variables and the observed and predicted response

Run	Yeast extract	Na_2HPO_4	Inoculum size	Endoglucanase activity (U/ml)	
				Observed	Predicted
1	8.47	3.24	4.19	462.19	439.98
2	5.50	4.05	3.00	388.12	416.30
3	5.50	2.05	3.00	423.06	466.91
4	5.50	2.05	3.00	460.45	466.91
5	5.50	0.05	3.00	639.11	622.76
6	8.47	3.24	1.81	435.46	456.94
7	8.47	0.86	4.19	543.57	558.43
8	5.50	2.05	3.00	462.17	466.91
9	5.50	2.05	3.00	481.68	466.91
10	2.53	3.24	4.19	326.45	296.23
11	5.50	2.05	1.00	448.73	424.51
12	2.53	0.86	4.19	306.84	277.00
13	2.53	0.86	1.81	329.17	343.01
14	0.50	2.05	3.00	84.03	121.29
15	5.50	2.05	5.00	318.69	354.74
16	8.47	0.86	1.81	699.83	721.69
17	5.50	2.05	3.00	486.76	466.91
18	2.53	3.24	1.81	239.16	215.94
19	10.50	2.05	3.00	586.03	560.60
20	5.50	2.05	3.00	489.38	466.91

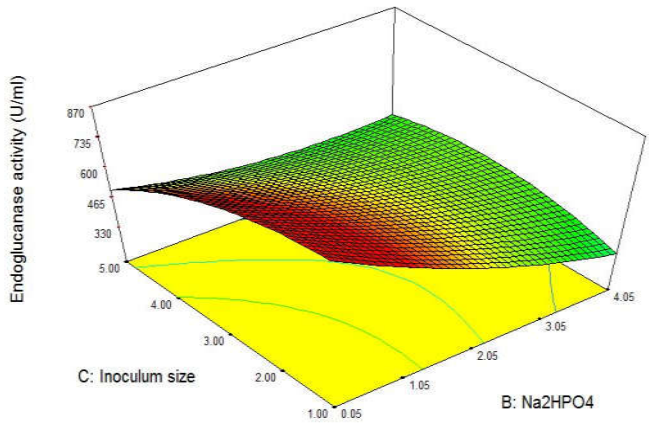
Table 4.8: Analysis of variance (ANOVA) of response surface quadratic model for the production of endoglucanase by *Bacillus subtilis* MU S1

Source	Sum of squares	df	Mean square	*F* value	*p*-value Prob > F	
Model	3.607E+005	9	40073.39	32.65	< 0.0001	significant
A-Yeast extract	2.330E+005	1	2.330E+005	189.79	< 0.0001	
B-Na_2HPO_4	51452.93	1	51452.93	41.92	< 0.0001	
C-Inoculum size	5875.59	1	5875.59	4.79	0.0535	
AB	9477.20	1	9477.20	7.72	0.0195	
AC	4728.30	1	4728.30	3.85	0.0781	
BC	10702.58	1	10702.58	8.72	0.0145	
A^2	28584.89	1	28584.89	23.29	0.0007	
B^2	4987.21	1	4987.21	4.06	0.0715	
C^2	10760.74	1	10760.74	8.77	0.0143	
Residual	12274.58	10	1227.46			
Lack of Fit	9171.17	5	1834.23	2.96	0.1297	not significant
Pure Error	3103.40	5	620.68			
Cor Total	3.729E+005	19				

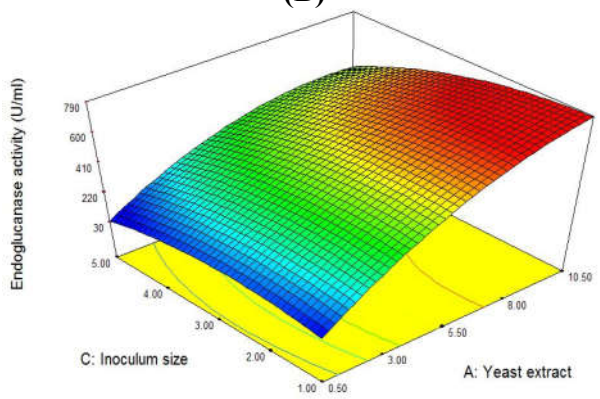
Three-dimensional response surface plots generated by the analysis were used to demonstrate the individual and interactive effect of the media components and predict their optimal levels (Figure 4.4).The plot shown in Figure 4.4A depicts the interaction between yeast extract and Na_2HPO_4. The endoglucanase activity increases with increasing concentration of yeast extract to some extent, above which there is no increase in activity. Whereas, Na_2HPO_4 was essential at a concentration slightly lower than the intermediate, for enhanced enzyme activity. Figure 4.4B shows the interaction between Na_2HPO_4 and inoculum size. An inoculum volume close to the intermediate level showed higher activity. These two plots showed strong interaction between components as indicated by $p < 0.05$. Figure 4.4C describes the interaction between yeast extract and inoculum size. The graph for yeast extract is ascending and for inoculum size is descending.



(A)



(B)



(C)

Figure 4.4: Response surface graph showing interaction effects between concentrations of selected variables **(A)** yeast extract and Na_2HPO_4 **(B)** Na_2HPO_4 and inoculum size **(C)** yeast extract and inoculum size

4.5.4.3. *Validation of the model*

The adequacy of the model was verified by carrying out set of experiments using the combinations of independent variables predicted for maximum response (Table 4.9). The maximum endoglucanase activity predicted by the model using the optimum concentrations of selected components (8.38 g/l yeast extract, 0.88 g/l Na_2HPO_4 and 2.05 % of inoculum) and 1 % arecanut husk as sole carbon source was 710.26 U/ml. This was in agreement with the experimental yield of 713.62 U/ml, which verifies the validity of the model.

Table 4.9: Validation of the model

Run	Yeast extract	Na_2HPO_4	Inoculum size	Endoglucanase activity (U/ml)	
				Observed	Predicted
1	8.38	0.88	2.05	713.62	710.26
2	8.44	0.90	2.06	711.03	708.32
3	8.43	0.90	2.04	705.63	708.48
4	8.13	0.89	1.92	689.33	703.67
5	8.19	0.91	1.95	683.84	701.90

4.5.5. Fold increase

A 2.2 fold increase in endoglucanase production was observed after statistical optimization of arecanut husk medium.

4.6. DISCUSSION

Over the past few years, there has been an increasing shift towards utilization of lignocellulosic wastes as the sole source of carbon for cost-effective enzyme production. The peculiar composition of these wastes

makes them an interesting source of various hydrolytic enzymes. In the present study, few locally available and less exploited wastes obtained from pepper, arecanut, tamarind, banana and jackfruit were used as carbon source.

From the results of earlier chapter it can be concluded that endoglucanase production by *Bacillus subtilis* MU S1 is highly dependent on media composition. So as to increase endoglucanase production three new formulations were tried, among which M1 and M2 showed 1.5 and 1.1 fold increase in activity as compared to the original medium. M3 displayed lower enzyme production efficiency than the original. The presence of new components such as Na_2HPO_4, $MnSO_4$, KCl, and $CaCO_3$ may be the reason for the increase in endoglucanase production in M1.The presence of high concentration of $FeSO_4$ in M2 must be the reason for the decrease in activity compared to M1. The absence of Na_2HPO_4 and presence of $FeSO_4$ could be the cause of low endoglucanase activity in M3. Ferrous sulphate is a source of iron and required at low concentrations. Higher concentration of iron used in the media is thought to cause acidification as opined by Jamal et al. (2012). Earlier studies have reported a positive (B.Sasirekha and B.J.Vishal 2017) and negative effect (Deka et al. 2011) of $FeSO_4$ on cellulase production by *Pseudomonas* spp. and *Bacillus subtilis* AS3 respectively. Disodium hydrogen phosphate, manganese sulphate, potassium chloride and calcium carbonate are sources of phosphorus, manganese, potassium and calcium respectively. These components are essential as they function as cofactors of many enzymes.

The newly formulated media was used to find the efficiency of agro-wastes as alternative carbon source. All the agro-wastes used supported the production of endoglucanase by the *B.subtilis* MU S1. Arecanut husk enhanced the production of endoglucanase, whereas the other substrate showed reduced production compared to control. The activities obtained from other wastes were in the order, TH > PW > BP > JOR. The difference in endoglucanase production by different wastes may be attributed to various factors such as cellulose content, heterogeneity and complexity of the material, accessibility to cellulose, sugar content and growth promoting factors (Goyal and Soni 2014). Lazim et al. (2014) reported the presence of complex polymers like cellulose (50.21 %), lignin (29.34 %) and hemicelluloses (13.16 %) in arecanut husk. The high cellulose content of arecanut husk may be the reason for high activity. The polymers like lignin and hemicelluloses combine with cellulose making it inaccessible to the enzyme. But, as *B. subtilis* MUS1 has the ability to produce various hydrolytic enzymes (mainly xylanase, ligninase, pectinase), it may have assisted in degradation of these complex polymers and exposed cellulose to cellulases. Also, the sugars released from these polymers may act as inducers further increasing endoglucanase production.

Short incubation times are desirable during enzyme production as it decreases contamination, operating cost and increases the profitability on an industrial scale. The maximum endoglucanase production in all the agro-waste media was recorded at 48 h. Similar incubation time was recorded for a strain of *Bacillus subtilis* during its growth on different agricultural residues (Roopa et al. 2017). Longer incubation times of 3

days and 6 days were reported by Padilha et al. (2015) and Kumar et al. (2009a) respectively, for endoglucanase production by different *Bacillus* spp. using agro-waste medium.

The ability of the strain to produce hydrolytic enzymes on AH medium was tested at 48 h of incubation. It was found that all hydrolytic enzyme tested could be produced using AH as the sole carbon source. The endoglucanase production from AH medium was optimized for using statistical methods. By Plackett-Burman design, three components (yeast extract, Na_2HPO_4 and inoculum size) with 100 % confidence were selected. Among the factors selected yeast extract served as the nitrogen source. Earlier studies on this strain had shown its preference towards organic nitrogen source. Works of Acharya and Chaudhary (2012) and Ray et al. (2007) showed preference of organic nitrogen sources for cellulase production by various *Bacillus* species. Incorporation of organic nitrogen sources like yeast extract and peptone to pretreated wheat bran medium has reported to increase the endoglucanase production by *Halomonas* sp. strain PS47 (Shivanand et al. 2013). However, Goyal et al. (2014) recorded enhanced endoglucanase production by *Bacillus* sp. *313SI* on the addition of inorganic nitrogen sources (ammonium sulphate and ammonium nitrate) to rice straw medium. Sodium dihydrogen phosphate was found to have an effect on endoglucanase production. It is a source of phosphorus and is needed for nucleic acid synthesis and also helps in functioning of many proteins. The size of inoculum is an important factor in any fermentation process. According to Abusham et al. (2009), initial inoculum size must be highly controlled, to ensure high enzyme production in a limited volume of medium. Among the other

constituents of AH medium, $(NH_4)_2SO_4$ and KCl had a positive effect on endoglucanase production whereas NaCl, $CaCO_3$, KH_2PO_4 and $MnSO_4$ had negative *t* value, but none of them had a significant effect on endoglucanase production.

The optimal levels of the selected components were determined by CCD. The ANOVA results of the model showed high *F*-value, low *p*-value, non-significant lack of fit, determination coefficient close to 1 and adequate precision greater than 4. From the results, it was confirmed that the proposed model was valid. Three-dimensional response surface plots were generated to determine the interactive effect of the selected components. It was observed that a high concentration of yeast extract and lower than intermediate concentration of Na_2HPO_4 showed high activity. Earlier studies on endoglucanase production by a *Bacillus* sp. showed that when organic sources like peptone were used as nitrogen source high activity was observed at a concentration of 0.5 % compared to 1 %. Whereas, inorganic nitrogen source, ammonium sulphate was required at 1 % concentration for high enzyme production using different agro-wastes (Hemavathy E et al. 2016). An inoculum size close to intermediate showed highest activity. The activity initially increased with increasing inoculum volume, but decreased after a maximum was attained. At the optimal point, equilibrium is maintained between the biomass and availability of substrates. But as the biomass increases, the competition for carbon source increases, nutrient availability decreases thereby reducing the metabolic activity of the cells. This causes overall decrease in growth and enzyme production (Singh and Kaur 2012). According to Lugani et al. (2015), high inoculum density affects the

length of stationary phase and leads to accumulation of toxic products and consequent loss of activity. Their observations were in par with our results. Inoculum size of 2 % and 1 % yeast extract was optimum for endoglucanase production by *Bacillus subtilis* K-18 (Irfan et al. 2017). After statistical optimization, a 2.2 fold increase in endoglucanase production was observed in a medium containing 1 % arecanut husk, 8.38 g/l, yeast extract, 0.88 g/l Na_2HPO_4 and 2.05 % of inoculum.

4.7. CONCLUSION

In the present study, the potential of five rarely used agro-wastes for endoglucanase production by the strain *Bacillus subtilis* MU S1 was examined. Among this arecanut husk emerged as a potent substrate and was found to produce all the hydrolytic enzymes tested. The production of endoglucanase from AH medium was enhanced by 2.2 fold using statistical methods. The study revealed the effective utilization of agro-wastes as a cheap carbon source for endoglucanase production. Exploitation of agro-wastes for enzyme production not only increases the economic value of the waste but also helps in waste management.

SREENA C. P. “IMPROVEMENT OF BIOCATALYTIC EFFICACY OF ENDOGLUCANASE FROM INDIGENOUS STRAIN, BACILLUS SUBTILIS MU S1 AND ITS APPLICATION IN SACCHARIFICATION OF AGRO-WASTES”. THESIS. DEPARTMENT OF LIFE SCIENCES, UNIVERSITY OF CALICUT, 2018.

5.1. INTRODUCTION

Endoglucanase is the major cellulase that hydrolysis the glycosidic linkages and releases oligosaccharides of varying lengths. It is considered to be more effective in cellulose degradation as compared to exoglucanase and β-glucosidase. Due to its immense potential, endoglucanases have been extensively used in various industries, particularly in production of biofuels by utilization of cellulosic biomasses. However, for industrial applications, large quantities of highly active enzymes are required. Enzymes with unique properties may be obtained from natural isolates, but its commercial production is limited by factors such as low yield and dependence on media components. Moreover, as cellulase is an inducible enzyme its expression depends largely on the presence of cellulosic substrate (Lambertz et al. 2014).

In order to overcome the limitations, recombinant DNA technology was implemented for the commercial production of a high level of enzymes. Besides this, it is used to produce enzymes from pathogenic and/or uncultivable microorganisms or to improve the activity and stability of enzymes by protein engineering. Currently, about 90 % of industrial enzymes available are recombinant proteins (Adrio and Demain 2014). Selection of the recombinant host is a vital factor in the success of rDNA technology. *E.coli* is the most commonly used bacterial expression host as its genome is well characterized and easy to manipulate. Moreover, a large quantity of strains and vectors are available commercially and the organism has the ability to express a high level of the recombinant gene, that makes about 50 % of the total protein (Baneyx 1999).

Cellulase enzymes belong to glycoside hydrolases which catalyze the hydrolysis of glycoside bond that links two or more carbohydrate molecules or a carbohydrate and non-carbohydrate molecule. They are grouped into different families based on the amino acid sequence similarity (Bernard et al. 1998b). Endoglucanase activity is found in many GH families among which proteins of family GH5, 7, 8, 9 and 44 are most active in cellulose hydrolysis (Yennamalli et al. 2011). The cellulases from *Bacillus* spp. belongs to GH5 and is made up of a catalytic domain and carbohydrate/cellulose binding domain. The availability of whole genome sequence of *Bacillus subtilis* allows direct manipulation of the desired gene. The cloning and overexpression of endoglucanase gene from *Bacillus subtilis* MU S1 in heterologous host like *E.coli* could be beneficial for direct industrial applications.

5.2. OBJECTIVES

The aim of this chapter was to clone endoglucanase gene (Egl) of *Bacillus subtilis* MU S1(Sreena et al. 2016) and express it in *E. coli* BL21 (DE3). The enzyme obtained was purified and characterized. The protein structure of the endoglucanase was modelled from the deduced amino acid sequence by using bioinformatics tools.

5.3. REVIEW OF LITERATURE

Cellulolytic enzymes with industrially important properties like stability under extreme conditions of pH, temperature, salinity have been obtained from natural isolates. But the low level of production often limits

its industrial application. Cloning and expression of the gene in industrially suitable host organism is one of the best approaches to obtain a high level of enzyme production. Cloning of a gene, besides increasing the level of enzyme production gives an insight into the structure and function relationship of enzyme which in turn paves way for protein engineering.

5.3.1. Cloning and expression of endoglucanase

The choice of expression vector and host is imperative in the success of rDNA technology. Selection of a suitable host is essential as each host has its own unique feature and a better understanding of the host can lead to the production of high titers of the desired product. Both bacteria and yeast have been used as recombinant hosts for heterologous expression of cellulases (Jung et al. 2012).

5.3.1.1. *Heterologous endoglucanase expression in bacteria*

Bacteria are the most preferred expression system for high level production of enzymes due to reasons like well-characterized genetics, availability of a huge number of vectors and mutant strains and also rapid growth on inexpensive substrates (Terpe 2006). But, they are unable to express large proteins and proteins requiring post-translational modifications. To date, many different bacteria both gram negative (such as *Escherichia coli* and *Zymomonas mobilis*) and positive (*Clostridium* and *Bacillus*) have been used as host organism for enzyme production. Among them, *E.coli* continues to be the most attractive host due to its short generation time, quick and easy overexpression of the recombinant enzyme, low cost and easy handling compared to other hosts (Gopal and

Kumar 2013). Table 5.1 gives a list of endoglucanase cloned into *E.coli*. However, few researchers have encountered difficulties in the expression of cellulase in *E.coli*. Meinke et al. (1991), Fierobe et al. (1991) and Mittendorf and Thomson (1993) observed degradation of linker sequence from endoglucanase of *Cellulomonas fimi, Clostridium cellulolyticum* and *Clostridium* sp. (*C. longisporum*) respectively when expressed in *E.coli*. Formation of inclusion bodies was reported by Jamaluddin and Salleh (2012).

As all proteins are not expressed equally well in *E.coli*, different approaches have been adopted to increase the expression and solubility of expressed proteins. The high level expression can be obtained by changing the vector, the host strain, culture conditions of recombinant host strain, the gene sequence without changing the functional domains and finally co-expression of other genes (Gopal and Kumar 2013).

5.3.1.1.A. *Vector*

The main components of a vector are the origin of replication, promoter, multiple cloning sites (MCS), terminators, selection markers, and the fusion protein. Of these, selection of appropriate promoter and fusion protein can significantly increase the enzyme production. The promoter to which the gene of interest is linked should be strong enough for accumulating about 10-30 % protein, should have low basal expression and be easy to induce. Some of the *E. coli* promoters reported were *lac, trp, tac, trc, ara,cspA, lpp, phoA, recA, tetA,* T7, T7 *lac* operator, T3-*lac* operator and T5-*lac* (Joseph et al. 2015). Although *E.coli'*s own promoters like *lac, trp, tac* have been used, T7 systems are

the most powerful and popular promoters because they produce the desired protein to a level about 50 % of the total cell protein (Baneyx 1999).

Often short peptides (tags) or fusion proteins are attached to the N- or C- terminal of the heterologous protein. Besides increasing the expression and solubility of the recombinant protein, affinity tags make their detection and purification easier. Some of the commonly used tags are poly-His, poly-Arg, FLAG, c-Myc, and Strep II- tags (Terpe 2003). Commercial antibodies are available for detection of the tags. The 6xHis affinity tag is the most widely used tag as it is small, less immunogenic, uncharged at pH 8.0. Moreover, it does not affect secretion, compartmentalization, protein folding, structure and function of the protein. Also, fusion proteins with affinity tags can be easily purified by affinity chromatography. Porath et al. (1975) used immobilized metal affinity chromatography for the first time to purify proteins using chelating agent iminodiacetic acid (IDA). Variety of proteins and peptides were purified using IDA charged with metal ions like Ni^{2+}, Co^{2+}, Zn^{2+}. But low yield and contamination problems limited its use. Later QIAGEN developed nitrilotriacetic acid (NTA) - agarose resins for His- tagged protein purification. These bind the His-tagged protein more tightly and allow quick and one step purification.

Non-peptide fusion proteins have an extra advantage as they increase the solubility by acting as chaperons (Hammarström et al. 2002). The most commonly used tags are glutathione *S*-transferase (GST) (Smith and Johnson 1988), thioredoxin (Trx) (Lavallie et al. 1993), ubiquitin (Baker 1996) maltose-binding protein (MBP) (Kapust and Waugh 1999),

N-utilization substance protein (NusA) (Davis et al. 1999), and SUMO (Small ubiquitin modifier) (Butt et al. 2005). The fusion proteins MBP, NusA and Trx display the best solubility but the solubility varied when the tag was removed. Therefore smaller and highly soluble tags are preferred. Lately, an 8-kDa calcium binding protein Fh8 obtained from the parasite *Fasciola hepatica* showed better solubility and retained solubility even after tag removal (Costa et al. 2013). For further biochemical and structural studies of cloned protein, the fusion tag must be removed. This can be done by enzymatic and chemical means. In case tag removal by enzymatic cleavage, the expression vectors itself have sequences that code for protease cleavage sites downstream of the gene coding for the tag. Enterokinase, factor Xa, thrombin, and the tobacco etch virus (TEV) protease have been effectively used to remove peptide tags and fusion proteins (Jenny et al. 2003; Blommel and Fox 2007). The most commonly used chemical reagent for tag removal is CNBr (Blommel and Fox 2007).

Plasmid copy number is another factor that affects the level of protein expression. Usually, high copy number is related to high expression, but most of the time it causes metabolic burden which decreases the growth of bacteria and the level of plasmid produced. Thus moderate and low copy number plasmids are desired (Bentley et al. 1990). The most commonly used expression vectors are pET series: T7 promoter system, pMB1 ori, 15–60 copies per cell, Novagen (Bolivar et al. 1977), pQE vectors: T5 promoter system, ColE1 origin, 15–20 copies per cell, Qiagen (Lee et al. 2006), pUC series: *lac* promoter, pMB1 derivative as origin, 500-700 copies per cell, NEB (Minton 1984). pGEX (GST tag)

and pMAL (MBP tag) are also used, but the disadvantage is that the large size of fusion tag interferes with the product if not removed properly. Expression of dual recombinant protein is available using plasmid of pACYC and pBAD series having p15A ori and 10–12 copies per cell (Chang and Cohen 1978), whereas, the pSC101 plasmid can be used for triple expression of the protein.

5.3.1.1.B. *Host strain*

There is a variety of expression host available for *E.coli* depending on the purpose. The strain BL21 (DE3) and derivatives of K-12 lineage strains are widely used for initial protein expression. BL21 (DE3) strain was developed by Studier and Moffatt (1986) after several modifications to B line. BL21 (DE3) has a T7 system. In the strain λDE3 prophage containing the T7 RNA polymerase under the *lac*UV5 promoter is inserted into the chromosome of BL21. The strain is deficient in *Lon* and *OmpT* protease which prevents it from degrading the foreign protein. Other BL21 derivatives used are BL21 (DE3) pLysS, Lemo21 (DE3), BL21-AI, C41 (DE3), Tuner (DE3), Rosetta2 and Rosetta pLysS, BL21 Codon Plus RIL and Codon Plus (DE3)–RIL/RIPL. All of them are induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). K-12 lineage strains are also used for expression of proteins. Origami is a K-12 derivative with mutations in thioredoxin reductase (trxB) and glutathione reductase (gor) genes, which enhances the disulphide bond formation in the cytoplasm (Derman et al. 1993). AD494 strains which are trxB mutant also favour disulphide bond formation. HMS174 which is a recA mutant strain of K-12 lineage shows a positive effect on plasmid stability (Marisch et al. 2013).

5.3.1.1.C. *Culture conditions of the recombinant host strain*

Altering the culture conditions of recombinant strain can to some extent lead to increased expression and solubility of the protein. Induction temperature, IPTG concentration and presence of inducers are some factors that influence the yield of recombinant protein. Prolonged induction at low temperature with decreased concentration of IPTG could increase the protein production (Steczko et al. 1991). Different researchers have tried to use chemical agents as inducers to accumulate chaperone which helps in proper folding of recombinant proteins. Some of the agents used as inducers were heat (Makrides 1996), osmolyte and salt stress (Diamant et al. 2003), benzyl alcohol (De Marco et al. 2005) and ethanol (Chhetri et al. 2015). In a study conducted by Liu et al. (2012), low temperature of 23–30 °C, IPTG concentration of 0.2 mM, EDTA 5 g/l and lysozyme 0.15 g/l enhanced the expression of endoglucanase from recombinant *Escherichia coli.* The source of the endoglucanase genes were *Bacillus akibai* III-3A and *Bacillus* sp. I-1A.

5.3.1.1.D. *Altering gene sequence without altering the functional domains*

Changing gene sequence is also a method to increase the expression level. Removal of signal peptide coding sequence from ORF and transmembrane or hydrophobic patch coding region have known to increase the expression and solubility of the recombinant protein. Formation of the secondary structure of mRNA also hinders the efficient translation of a gene. This can be prevented by optimization of gene sequence (Gopal and Kumar 2013).

5.3.1.1.E. *Co-expression*

Some proteins require counterparts for its stability. That is they are either not expressed or when expressed degrade in the absence of counterparts. In such cases, the genes coding for the counterpart protein, which may be a chaperone, increases the expression and solubility of the desired protein. Co-expression of multiple plasmids encoding different chaperone (de Marco and De Marco 2004) and single plasmid encoding single chaperone with the target protein could increase the solubility of the target protein.

Zymomonas mobilis has also been used as a host for endoglucanase gene expression. In 1988, Lejeune et al. cloned and expressed the endoglucanase gene *eglX* from *Pseudomonas fluorescens* var. *cellulose* in *Z. mobilis*. In the same year Yoon et al. (1988) expressed *Bacillus subtilis* endo-1, 4-glucanase gene in *Zymomonas anaerobia* but with much lower efficiency compared to *E.coli.* Other endoglucanase genes cloned in *Zymomonas mobilis* were from *Cellulomonas uda* CB4 (Misawa et al. 1988), *Erwinia chrysanthemi* (Brestic-Goachet et al. 1989), *Acetobacter xylinum* IFO 3288 (Okamoto et al. 1994) and *Enterobacter cloacae* (Vasan et al. 2011). There are very few reports on the use of Gram positive bacteria as host for endoglucanase production. Ahmad et al. (2013) expressed endoglucanase gene from *Clostridium phytofermentans* ISDg in *Bacillus subtilis* SCK6. Liu and Du (2012) were successful in over expressing endoglucanase gene from *Bacillus akibai* I-1 in *Bacillus subtilis* 168.

Table 5.1: List of endoglucanase genes cloned and expressed in *E.coli*

Wild type	Cloned gene	Expression Host	plasmid	Molecular weight (kDa)	Optimum conditions	Active against	Reference
Fibrobacter succinogenes AR1	-	*E.coli* HB101 and DH1	pGem-1, pGem-2, pGem7Zf+, and pGem7ZF	46.5	pH 5.0 temp-39 °C	acid-swollen cellulose, ball-milled CMC, filter paper, Avicel, lichenan, and xylan	Cavicchioli and Watson (1991)
Cellulomonas sp. *CelB7*	cegA	*E. coli* DH5α	pC72	36	pH 7.4 temp- 35°C	CMC, lichenin, crystalline cellulose and birchwoodxylan.	Fülöp et al. (1996)
Neocallimastix frontalis MCH3	celA	*E. coli* M15	pQE	50	pH 8.5 temp- 40°C	CMC, Avicel, xylan, lichenan	Fujino et al. (1998)
Eubacterium cellulosolvens 5	cel5A	*E. coli* BL21(DE3) and M15 (pREP4)	pCRT7-TOPO and pQE-30	127	-	CMC, ASC, oat spelt xylan, and lichenan	Yoda et al. (2005)
Bacillus subtilis strain I15	celI15	*E.coli* BL21 (DE3)	pET25b	52	pH 6.0 temp- 60°C	-	Yang et al. (2010)
Bacillus amyloliquefaciens PSM 3.1	EglII	*E. coli* BL21 (DE3)	pET-20b		pH 6.0 temp- 50°C		Nurachman et al. (2010)

Aspergillus fumigatus MKU1	*eng*61	*E. coli* BL21 DE3	pET30b	35.4 (predicted)	pH 5.0 temp-60°C		Meera et al. (2011)
Pseudomonas sp. MM15	Cel5M	*E. coli* strain BL21 (DE3)	pET28a+	50.6	pH 4.5 temp- 30 °C		Yang and Dang (2011)
Streptomyces sp. strain G12	CelStrep	*E. coli* strain BL21 CodonPlus (DE3) RP	pET28a	37	temp- 50°C	-	Amore et al. (2012)
Bacillus licheniformis ATCC 14580	bgl C	*E. coli* BL21 (DE 3)	pET-22b (+)	52.2	pH 6.0 temp- 60 °C	-	Aftab et al. (2012)
Pectobacterium carotovorum subsp. *carotovorum*	celB celC	*E. coli* DH5α	pTAC-MAT-TAG ®-2	29.5 40	-	-	Ibrahim et al. (2013)
Bacillus subtilis JS2004	-	*E. coli* BL21 (DE3)	pET-28a(+)	63	pH 9.0 temp 50 °C		Zafar et al. (2014)
Bacillus subtilis IARI-SP-1	Cel-SP1	*E. coli* BL21 (DE3)	pET-28a	55	pH 8.0 temp-50–60 °C	-	Pandey et al. (2014)
Salmonella enterica	-	*E.coli* (BL21 DE3)	pET 100	65	-	-	Aruna and Vijayarani (2014)
Bacillus sp. RP1	EG	*E. coli* TOP10	pGEM-T	55.2	pH 5.0 temp- 50 °C	-	Ramdani Moeis et al. (2014)
Bacillus subtilis UMC7	EG1	*E. coli* BL21 (DE3)	pET-30a(+)	56	pH 6.0 temp- 60 °C	CMC, avicel, cotton linter, filter paper	Chuan Wei et al. (2015)

Acidothermus cellulolyticus 11B	AcCel12B	*E. coli* BL21-CodonPlus (DE3)-RIL	pET-20b	38.3	pH 4.5 temp- 75 °C	CMC (low viscosity and medium viscosity), Avicel® PH101, β-D-Glucan (barley), RAC.	Wang et al. (2015)
Paenibacillus sp. IHB B 3084	EG5C	*E. coli* BL21(DE3)	pET-28a(+)	62	pH 5.0 temp-40 °C		Dhar et al. (2015)
Citrobacter farmeri A1	EglC	*E. coli* BL21	pET22b	42	pH 6.5-8.0 temp- 30-40 °C	-	Bai et al. (2016)
Trichoderma virens ZY-01	EG	*E. coli* BL21(DE3)	pET-32a	39	pH 7.0 temp- 40 °C	-	Zeng et al. (2016)
Actinomyces sp. KNG 40	EG1	*E. coli* DH5α	pUC19	57.1 and 54.1	pH 6.0 temp- 55 °C	CMC, xylan, avicell, and cellobiose	Kim et al. (2016)
Serratia proteamaculans CDBB-1961	spr cel8A	*E. coli* M15 [pREP4]	pQE30	39	pH 7.0 temp- 40 °C	CMC, beechwoodxylan and birchwoodxylan	Cano-Ramirez et al. (2016)
Bacillus subtilis SB13	-	*E. coli* BL21 (DE3) Rosetta	pET30a	59	-	-	Guan et al. (2017)

5.3.1.2. *Heterlogous endoglucanase expression in yeast*

Yeast is the best eukaryotic expression system as it is easy to manipulate, produces a high level of expression, grows fast, leads to proper protein processing and post-translational modifications. The most commonly used yeasts are *Saccharomyces cerevisiae, Pichia pastoris,* and *Hansenula polymorpha* (Mattanovich et al. 2012). Li et al. (2011) expressed a neutral endoglucanase from *Volvariella volvacea* WX32 in *Pichia pastoris* GS115. The protein had a molecular weight of 42 kDa and was stable at a broad pH range of 6.0–9.0 and at a temperature of 55 °C or below. Pham et al. (2011) expressed endoglucanase gene from *Aspergillus niger* VTCC-F021 in *Pichia pastoris*. They obtained optimal enzymatic activity at a temperature of 55 °C and a pH of 5.0. The purified endo-1,4-β-glucanase of *Aspergillus usamii* E001 expressed in *P. pastoris* displayed the highest activity at pH 5.0 and temperature 60 °C (Shi et al. 2012). Valencia Jimenez et al. (2014) was successful in expressing endoglucanase from *Diabrotica virgifera virgifera* LeConte (western corn rootworm) in the same strain of *Pichia pastoris*. *Saccharomyces cerevesiae* has also been used as expression hosts. Endoglucanase gene from sources like white rot fungus *Irpex lacteus* strain MC-2 (Toda et al. 2005) and *Trichoderma viride* AS 3.3711 (Huang et al. 2012) have been expressed in *S. cerevisiae.* Akbarzadeh et al. (2013) observed that when the biochemical properties of recombinant endoglucanase II of *Trichoderma reesei* in *Hansenula polymorpha* and *Pichia pastoris* were compared both were found to be good hosts for expression and production of thermostable endoglucanase.

5.3.2. Cloning and expression of GH 5 family

Currently, the most significant application of cellulase enzyme is in biofuel production using cellulosic biomass. For complete conversion of cellulosic substrate to glucose, the synergistic action of three enzymes, i.e. endoglucanase, exoglucanase and β–glucosidases is required. Cloning of multiple genes in host organism is technically challenging and will affect the expression level. Therefore, efforts are being made to identify and clone genes with multiple cellulolytic functions (Jung et al. 2012).

Over the years endoglucanase enzymes belonging to different glycoside hydrolase families have been cloned and expressed. The family 5 glycoside hydrolases exhibit wide variation in specificity and hydrolytic activity and therefore have become the most prominent endoglucanase in biomass conversion cocktails. It contains around 20 experimentally defined enzyme activities among which cellulases, xylanases, mannanases, galactanases, and xyloglucanases are the most relevant in the conversion of biomasses. It was the first cellulase family to be described and was known as "cellulase family A". Now it has become the largest family among the glycoside hydrolases (Aspeborg et al. 2012). They classified catalytic module of GH5 family and found that about 80 % of it could be grouped into 51 subfamilies.

Glycoside hydrolases belonging to GH5 family have been cloned from various sources. GH5 gene from *C. thermocellum*, expressed in *E. coli* BL21 (DE3) and BL21(C43) had both endo-cellulase and exo-cellulase activities (Brumm et al. 2015). In the same

year Zhang et al. (2015) expressed endoglucanase from *Thermobifida halotolerans* YIM 90462(T) in *E. coli* BL21 (DE3). Other GH5 family endoglucanases that were expressed in *E. coli* were from *Bacillus subtilis* strain I15 (Yang et al. 2010), *Bacillus amyloliquefaciens* PSM 3.1 (Nurachman et al. 2010), *Bacillus subtilis* JS2004 (Zafar et al. 2014), *Bacillus subtilis* IARI-SP-1 (Pandey et al. 2014), *Bacillus* sp. RP1 (Ramdani Moeis et al. 2014), and *Bacillus subtilis* UMC7 (Chuan Wei et al. 2015). The GH5 gene from *Volvariella volvacea* WX32 was successfully expressed by Li et al. (2011) in *Pichia pastoris.*

5.3.3. Characterization of endoglucanase

Characterization of an enzyme is an inevitable part of enzyme research. Several factors like the temperature, pH, presence of inhibitors or activators, enzyme and substrate concentration affect the rate of an enzymatic reaction. Various physical and chemical properties (such as molecular weight, optimum pH, temperature, the presence of metal ions, inhibitors and activators) of cloned endoglucanase have been studied.

Molecular weight, pH and temperature depend on the organism and vary from organism to organism. Most of the *Bacillus* spp. has a molecular weight in the range of 30-100 kDa. A lower molecular weight of 16.9 kDa was observed for endoglucanase from *Bacillus cereus* (Nema et al. 2016). A molecular weight in the range of 55-56 kDa was observed in some strains of *Bacillus subtilis* (Pandey et al. 2014; Chuan Wei et al. 2015). A higher molecular weight of 80 kDa was observed in alkalophilic *Bacillus* sp. HSH-810 (Kim et al. 2005).

Near neutral to alkaline pH (5.0-10.0) was found to be optimum for endoglucanase from different *Bacillus* sp. As that for molecular weight and pH, optimum temperature also depends on the bacteria. An optimum of 50-60 °C was observed by endoglucanase obtained from various *Bacillus* spp. (Yin et al. 2010a; Chuan Wei et al. 2015).

Metal ions play a crucial role in the biological function of enzymes. They can either act as enhancers or inhibitors of enzyme activity. The records on the effect of metal ions on endoglucanase activity of various *Bacillus* spp. show that Mg^{2+}, Ca^{2+}, Mn^{2+} and Co^{+2} generally enhance endoglucanase activity whereas Hg^{2+}, Cd^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Ni^{2+} and Cr^{2+} inhibit endoglucanase activity. In some cases, Fe^{3+} was known to activate and Mn^{2+} known to inhibit endoglucanase activity (Chuan Wei et al. 2015). Researchers have also tried the effect of certain chemical detergents on endoglucanase activity. The chemicals like SDS, p-CMB, DTT, Tween-20, Tween-80, Triton X-100, phenylmethanesulfonyl fluoride (PMSF), urea were found to inhibit endoglucanase activity (Yin et al. 2010a). In order to know the suitability of endoglucanase in detergent industries, Sadhu et al. (2013) studied the effect of some commercial detergents on endoglucanase activity.

The specificity of endoglucanase enzyme towards different substrates like avicel, filter paper, p-nitrophenyl α-d-glucopyranoside, xylan, chitin, cotton linter, starch and pectin was tried by Chuan Wei et al. (2015). The specificity of endoglucanase towards different substrates increases its applicability in various industries.

5.4. MATERIALS AND METHODS

5.4.1. Bacterial strains and plasmid

The *E.coli* strains used for cloning and expression were *E.coli* DH5α and *E.coli* BL21 (DE3) respectively. The plasmid pET 22b (+), used for cloning was obtained from Promega, USA.

5.4.2. Cloning and transformation of endoglucanase gene into *E.coli* DH5α

5.4.2.1. Primers

Primers for amplification and cloning of endoglucanase (Egl) gene without signal sequence were designed using a published sequence of *Bacillus subtilis* subsp. subtilis str. 168 chromosome as the template. These primers (Table 5.2) with restriction sequences for *Xba* I and *Xho* I in forward and reverse primer respectively were synthesized and supplied by Eurofins Genomics, Bangalore, Karnataka.

Table 5.2: Sequences of the designed primers

Primer	Restriction enzyme site	Sequence	Bases	T_m (^oC)	GC %
Forward	*Xba* I	5'-GCTCTAGAATGAAACGGTCAATCTCTA-3'	27	56.1	40.7
Reverse	*Xho* I	5'-CTCGAGATTTGGTTCTGTTCCC-3'	22	55.6	50

5.4.2.2. Genomic DNA extraction

Bacillus subtilis MU S1 was grown overnight in LB broth at 37 °C and total genomic DNA was isolated by Unal et al. (1992) method with some modifications. In brief, the culture was pelleted by centrifugation at 5000 rpm for 5 min at 4 °C and the supernatant discarded. The cells were resuspended in 100 µl lysozyme (100 mg/ml) and incubated at 37 °C for 1 h. To this 20 µl of proteinase K (20 mg/ml) and 250 µl buffer (0.1 M Tris- HCl, pH 7.5) was added and incubated at 37 °C for 30 min and then placed in boiling water bath for 10 min. This mixture was centrifuged at 5000 rpm at 4 °C for 5 min and the supernatant was transferred into a sterile eppendorf tube. To this equal volume of isopropanol was added, mixed well and centrifuged at 12,000 rpm for 10 min for pelleting DNA. The pellet obtained was desalted with 200 µl of 70 % ice cold ethanol and regained by centrifugation. The DNA was dried and dissolved in 100 µl nuclease free water. The concentration and purity of the isolated DNA was quantified using NanoDrop 2000c spectrophotometer (Thermo Scientific). The DNA having ratio between 1.8 and 2.0 was considered of good purity and was used as a template for amplification. Agarose gel electrophoresis was also performed to qualitatively examine the DNA.

5.4.2.3. Annealing temperature standardization using gradient PCR

Gradient PCR was performed to determine the optimum annealing temperature. A range between 51 °C – 59 °C was selected

and the annealing was checked at following temperatures (51.7, 52.4, 53.3, 55.3, 56.2, 57.9, 58.8 °C). PCR amplification was done in 50 µl reaction volume containing 25 µl OrionX Taq PCR mix (2X), 2 µl each of forward and reverse primer, 2 µl of genomic DNA (120.29 ng/µl) and final volume was made upto 50 µl with nuclease free water. The PCR was performed in eppendorf thermal cycler as per protocol in Table 5.3.

Table 5.3: PCR amplification profile

Initial denaturation	Denaturation	Annealing	Extension	Final Extension
94 °C	94 °C	52.4 °C	72 °C	72 °C
2 min	30 sec	40 sec	2 min	5 min
	35 cycles			

5.4.2.4. Purification of PCR product

The PCR product was purified by PCR purification kit (Promaga-wizard SV gel & Gel PCR cleaning up system). To 300 µl of the PCR product an equal volume of Membrane Binding Solution was added and transferred into it an SV minicolumn placed in collection tube, and incubated at room temperature for 1 min. This was centrifuged at 16,000 g for 1 min and flowthrough discarded. To the column, 700 µl membrane wash solution was added and centrifuged at 16,000 g for 1 min. Next, 500 µl of the same solution was added and centrifuged at 16,000 g for 5 min. To allow the residual ethanol to evaporate, the flowthrough obtained in previous step was discarded and the column assembly centrifuged for 1 min with the microcentrifuge lid open (or off). The minicolumn was the transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of nuclease-free water

was added into the minicolumn. This was incubated at room temperature for 1 min followed by centrifugation at 16,000 g for 1 min. The minicolumn was discarded and DNA stored at –20 °C. The purity of PCR product before and after PCR purification was checked using NanoDrop.

5.4.2.5. Plasmid isolation

The plasmid vector used for the study was pET-22b (+) which is an expression vector. The pET-22b (+) vector carries an N-terminal *pelB* signal sequence for potential periplasmic localization, plus optional C-terminal His•Tag® sequence. It is a 5493 bp plasmid (Figure 5.1). Plasmid transformed in BL 21 was isolated by QIAGEN® Plasmid Midi Kit using Quick-Start protocol.

For midi prep, a 100 ml LB broth containing 100 µl carbinecillin (100 mg/ml) was inoculated with culture and incubated overnight. The culture was pelleted by centrifugation at 6000 g for 15 min at 4 °C and the pellet was resuspended in 4 ml Buffer P1. To this 4 ml, Buffer P2 was added, mixed by vigorously inverting 4–6 times, and incubated at room temperature (15–25 °C) for 5 min. The solution turns blue due to LyseBlue reagent. Prechilled Buffer P3 (4 ml) was added, mixed thoroughly by vigorously inverting 4–6 times and incubated on ice for 15 min. The solution was mixed until it turns colourless. The solution was centrifuged at ≥ 20,000 g for 30 min at 4 °C. The supernatant was applied to QIAGEN-tip 100 column preequilibrated with 4 ml Buffer QBT and allowed to enter the resin by gravity flow. The QIAGEN-tip was then washed two times with 10 ml Buffer QC. The DNA was eluted with 5 ml Buffer QF into a clean 15 ml vessel and precipitated by adding 3.5 ml (0.7 volumes) room-

temperature isopropanol. It was mixed and centrifuged at ≥ 15,000 g for 30 min at 4 °C. The supernatant was carefully decanted, the DNA pellet washed with 2 ml room-temperature 70 % ethanol and centrifuged at ≥ 15,000 g for 10 min. The supernatant was decanted and pellet air dried for 5–10 min. The DNA was redissolved in 100 µl nuclease free water. The purity of the plasmid was checked using NanoDrop.

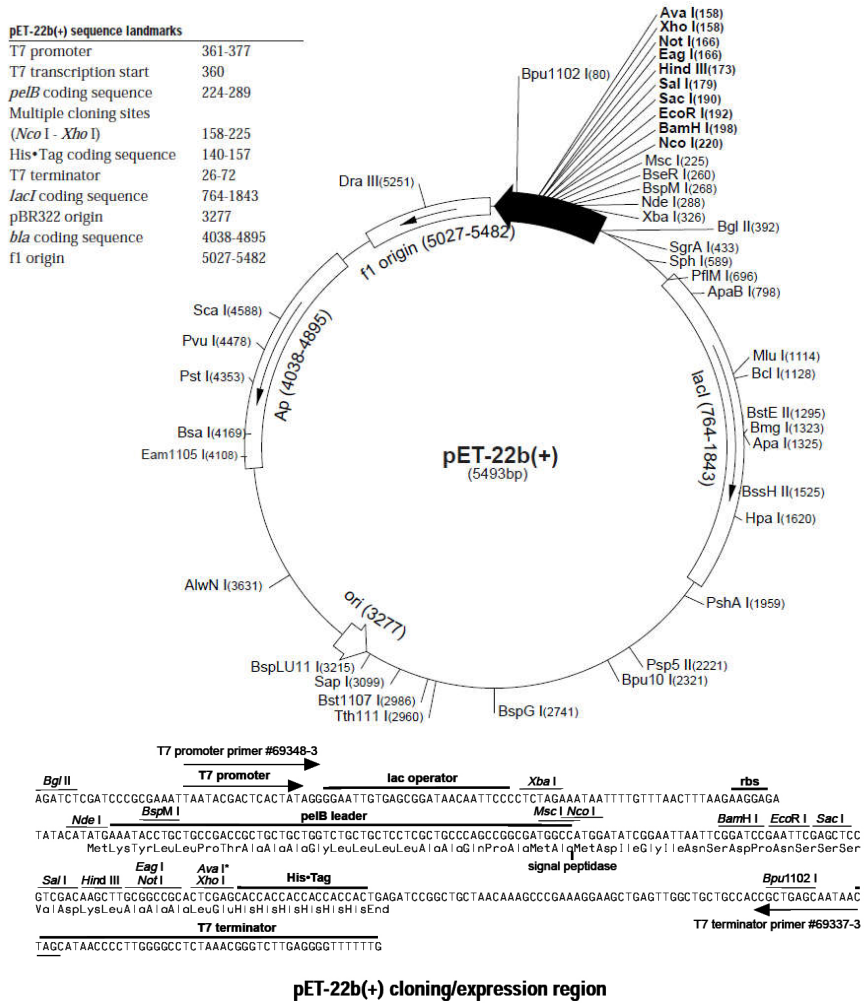


Figure 5.1: Vector diagram and sequence of expression vector pET 22 b (+)

5.4.2.6. Restriction digestion of vector and insert

Restriction digest is most commonly used as part of the process of the molecular cloning of DNA fragment into a vector. Single digest of the vector (pET22b) was done using the restriction enzymes *Xba* I and *Xho* I individually and for double digest, a combination of both was used. All digests were made to a final volume of 20 µl (Table 5.4 a, b). The restriction enzymes and buffers were purchased from New England Biolabs. Double digested vector (pET22b) and the insert (purified PCR product) were prepared for ligation. Restriction was carried out in MJ mini Thermal cycler (BioRad) at 37 °C for 1 h followed by inactivated of enzymes at 65 °C for 20 min.

Table 5.4a: Restriction digestion of vector

Components	Uncut	Single cut		Double cut
		***Xba*I**	***Xho*I**	
Vector (µl)	0.4	0.4	0.4	0.4
10 X CutSmart buffer (µl)	2	2	2	2
Enzyme (µl)	-	1	1	1+1
NFW (µl)	17.6	16.6	16.6	15.6

Table 5.4b: Restriction digestion of Insert

Components	Double cut
Insert (µl)	1.6
10X CutSmart buffer (µl)	2
Enzyme (µl)	1+1
NFW (µl)	14.4

5.4.2.6.A. *Alkaline phosphatase treatment*

The vector was treated with alkaline phosphatase to prevent self-ligation thereby facilitating ligation of other DNA fragments into the vector. Alkaline phosphatase removes the 5' phosphates from vectors that have been cut with a restriction enzyme. Here the vector was treated with 2 µl Calf intestinal alkaline phosphatase (CIAP) after 1 h of restriction and incubated at 37 °C for 5 min. The enzymes were inactivated at 65 °C for 20 min.

5.4.2.7. Gel extraction

The restricted products were visualized on 1 % agarose gel stained with EtBr and the gel containing the region of interest was sliced with a clean scalpel and purified using a QIAquick Gel Extraction Kit (Qiagen). The gel slice was weighed in a clean tube. To 1 volume of gel, 3 volumes of Buffer QG was added and incubated at 50 °C for 10 min. The tubes were then vortexed every 2-3 min to help dissolve gel. It was incubated at 50 °C for an additional 5 min. To this 1 volume of isopropanol was added and mixed well. The sample was applied to the spin column placed in collection tube and centrifuged at 13,000 rpm for 1 min. To wash the column 750 µl Buffer PE was added and centrifuged at 13,000 rpm for 1 min. To remove any residual wash solution the centrifugation was repeated with empty column. The column was placed in a clean 1.5 microcentrifuge tube and eluted with Buffer EB. Thirty microlitre of EB was added to the centre of the column, incubated for 4 min and centrifuged at

13,000 rpm for 1 min. The concentration and purity of eluted DNA was checked using NanoDrop.

5.4.2.8. Ligation of vector and insert

Ligation of vector and insert DNA was performed in a total volume of 20 µl containing 5 U/µl of T4 DNA ligase, 10X T4 DNA ligase buffer (Thermo Scientific), insert DNA and 100 ng vector (Table 5.5). Two different molar ratios of vector: insert DNA were used (1:3 and 1:5). A mixture containing the vector alone without the insert was kept as control. Online ligation tool (http://www.insilico.uni-duesseldorf.de/Lig_Input.html) was used to calculate the concentration of insert in ligation mix. The ligation reaction was mixed and incubated at 16 °C overnight in MJ mini Thermal cycler (Bio- Rad).

Table 5.5: Components of ligation mixture (20 µl)

Components	Vector only	Vector + Insert	
		1:3	1:5
Vector (µl)	1.35	1.35	1.35
Insert (µl)	-	1.28	2.12
Buffer (µl)	2	2	2
Enzyme (µl)	1	1	1
NFW (µl)	15.65	14.37	13.53

5.4.2.9. Preparation of chemically competent *Escherichia coli* using calcium chloride method

The competent cells for introduction of recombinant plasmids were prepared using calcium chloride method. A loopful of *E. coli* DH5α culture from -80 °C (glycerol stock) was inoculated into 5 ml of LB broth and incubated overnight at 37 °C in an orbital shaker with an agitation of 200 rpm. From the overnight culture around 1 ml was added to 100 ml of LB broth and incubated at 37 °C with agitation of 200 rpm for 1 to 2 h until the OD reaches 0.35 to 0.4. The culture flask was chilled in ice for 20 min. The culture was then transferred aseptically to two precooled 50 ml centrifuge tubes and centrifuged at 6000 rpm for 8 min at 4 °C. The supernatant was discarded carefully and the pellet was resuspended gently in 2 ml ice cold 0.1 M $MgCl_2$ solution. These tubes were centrifuged at 6000 rpm for 10 min at 4 °C after incubation on ice for 20 min. The supernatant was discarded carefully and pellet resuspended gently in 2 ml of ice cold 0.1 M $CaCl_2$ solution. The tubes were kept on ice for 30 min, after which they were centrifuged at 6000 rpm for 10 min at 4 °C. The pellet was resuspended in 0.5 ml of 0.1 M $CaCl_2$ and 0.5 ml of 80 % sterile glycerol. Aliquots of 100 µl of competent cells were aseptically transferred in to 1.5 ml pre chilled microcentrifuge tubes and stored at -80 °C until use.

5.4.2.10. Transformation

For transformation, to 100 µl of competent cells about 10 µl of ligated product was added, tapped gently and incubated on ice for

30 min. Heat shock was given to the cells by placing the vials at 42 °C for 1-2 min. The vials were immediately returned to ice to chill for 5 min. To this 900 µl of LB broth was added and incubated at 37 °C in shaking incubator at 200 rpm for 1:30 h. After incubation, the vials were centrifuged at 5000 rpm for 5 min at 4 °C. After centrifugation, 900 µl was removed and the pellet was resuspended in remaining 100 µl of the medium. This suspension was spread on LB plates containing carbinecillin (100 mg/ml) and incubated at 37 °C overnight.

5.4.2.11. Colony PCR

After incubation few colonies were observed on the plate and the selected colonies were subjected to colony PCR for the confirmation of insert. The colonies were picked and suspended in 10 µl of 1X PBS. One microlitre of this suspension was used as a template for the PCR with the endoglucanase primers. Genomic DNA was used as the positive control. PCR was performed using the same protocol as mentioned above (Table 5.3) with initial denaturation of 7 min. The positive colonies were streaked on plates and also inoculated into 5 ml LB broth containing carbinecillin for plasmid isolation.

5.4.2.12. Plasmid isolation (Mini Prep)

Plasmid isolation was done with overnight culture of positive clone, using QIAGEN Plasmid Mini Kit using Quick-Start Protocol. Briefly, the cells were harvested using 6000 g for 15 min at 4 °C and resuspended in 0.3 ml Buffer P1 followed by addition of 0.3 ml Buffer P2. This was mixed by inverting 4–6 time and to this 0.3 ml, prechilled

Buffer P3 was added. The tubes were placed on ice for 5 min after mixing. It was centrifuged at 14,000–18,000 g for 10 min at 4 °C. The supernatant was applied to pre equilibrated QIAGEN-tip 20 column and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed twice with 2 ml Buffer QC. The DNA was eluted with 0.8 ml Buffer QF into a clean 2 ml vessel and precipitated by adding 0.56 ml (0.7 volumes) room-temperature isopropanol. It was mixed and centrifuged at ≥ 15,000 g for 30 min at 4 °C. The supernatant was carefully decanted and the DNA pellet washed with 1 ml room-temperature 70 % ethanol. It was centrifuged at ≥ 15,000 g for 10 min. The supernatant was discarded and pellet air dried for 5–10 min. The DNA was redissolved in 30 µl nuclease free water. The purity of the plasmid was checked using NanoDrop.

5.4.2.13. Confirmation of clone

5.4.2.13.A. *Restriction digestion of plasmid*

Confirmation of the positive clones was carried out by digestion of the plasmid with restriction enzymes in 20 µl reaction volume at 37 °C for 1 h followed by inactivation of enzymes at 65 °C for 20 min. Both single cut and double digestion of the isolated plasmid was done using *Xba* I and *Xho* I and the products were analyzed on 1 % agarose gel. Uncut and single cut vector were kept as control (Table 5.6).

Table 5.6: Restriction digestion of plasmid for clone confirmation (20 µl)

Components	Uncut vector	Single cut vector	Single cut insert	Double cut insert
DNA (µl)	0.25	0.25	1	1
Buffer (µl)	2	2	2	2
Enzyme (µl)	-	1	1	1+1
NFW (µl)	17.75	16.75	16	15

5.4.2.13.B. *PCR and sequencing*

PCR amplification and sequencing is another method for the confirmation of insert. The presence of the insert in multiple cloning sites was confirmed by amplification using T_7 forward and reverses primers which flank the multiple cloning site of pET 22b (+) vector. The product obtained was sequenced (SciGenom Labs Private Ltd., Cochin). The vector contamination was screened using VecScreen (NCBI) and manually deleted. The sequence was deposited in Genbank database.

5.4.3. In-silico characterization of endoglucanase

Similarity search of the sequence was done using nBLAST tool of NCBI. The amino acid sequence was deduced from nucleotide sequence using online translate tool, ExPASy. Physicochemical properties of the deduced protein such as molecular weight and theoretical pI were computed using Expasy's ProtParam Proteomics server. The signal peptide was predicted using SignalP 4.1. The domains in the protein were analyzed using InterProScan and the secondary structure composition was predicted using PredictProtein.

5.4.3.1. *3D structure prediction by homology modelling*

The protein was modelled using Modeller 9.18 (2017 Version) (Webb and Sali 2014). Firstly, the catalytic and non-catalytic domain were modelled separately using the A chain of 3PZT and 2L8A as template respectively. Later a single structure was modelled using a single template prepared by taking the 3D coordinates of the two domains (3PZT and 2L8A). After modelling, the protein was energy minimized as per the standard protocol by YASARA and Mod Refiner software. Then the models were visualized by UCSF Chimera. The models were validated and refined by Rampage (Ramachandran plot). The full structure of endoglucanase was superimposed with the template using Superpose/Dali and the RMSD value was predicted (Holm and Rosenstrom 2010).

5.4.4. Transformation and expression of recombinant plasmid in *E.coli* BL-21 (DE3)

5.4.4.1. Sub-cloning into expression host

The strain *E.coli* BL21 (DE3) was used as expression host. About 5 µl recombinant plasmid was transformed into *E.coli* BL21 (DE3) cells using the procedure mentioned in 5.4.2.10. From the colonies obtained four were randomly selected and colony PCR was performed as mentioned earlier in order to confirm transformation. The positive colonies were streaked on plates and also stored as glycerol stock.

5.4.4.2. Expression studies of endoglucanase

A single positive colony was inoculated into 10 ml broth containing 10 µl carbenicillin and incubated at 37 °C till the OD reached 0.8-1.2. This was used as starter culture for all inoculations. About 150 µl of the culture was inoculated into 50 ml LB broth supplemented with 50 µl carbenicillin and incubated on orbital shaker (200 rpm) at 37 °C. Production of endoglucanase by the recombinant cells was induced at an OD_{600} nm between 0.5-0.6 by addition of 1mM IPTG and incubated overnight (16 h). To estimate the production of recombinant protein, about 10 ml of culture was centrifuged at 12,000 rpm for 15 min at 4 °C. Endoglucanase activity was estimated in three fractions separately: (a) cell free supernatant (b) cell pellet suspended in 1 ml of 50 mM sodium citrate buffer (intracellular fraction in buffer) and (c) cell pellet with 1 ml of the supernatant (intracellular fraction in supernatant). To the resuspended pellets 10 µl of protease inhibitor was added and sonicated for 10 min with 15 s on and 10 s off cycle at amplitude of 50 % to lyse the cells. The suspension was centrifuged at 12,000 rpm for 15 min to get the protein in solution. The endoglucanase (CMCase) activity was determined by using CMC as substrate (Ghose 1987) and measuring the amount of reducing sugar (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of reducing sugar min^{-1}.

5.4.4.2.A. *Optimization of protein expression*

Protein expression was standardized by varying different parameters and performing endoglucanase activity. Initially, IPTG

concentration was varied. Different concentrations of IPTG (0.5, 1, and 1.5 mM) were tried for induction at 37 °C and incubated overnight. The optimized concentration of IPTG was used to standardize the temperature (25 °C, 30 °C and 37 °C) and the time of production (3, 16 and 24 h). Intracellular fraction in supernatant was used for assaying endoglucanase activity. Plate assay of the endoglucanase enzyme obtained after incubation at 25 °C at different time was performed and compared with the supernatant from *Bacillus subtilis* MU S1.

5.4.4.2.B. ***Purification of recombinant protein***

To facilitate purification, the recombinant protein was expressed with a His tag at its C-terminal in BL21. About 150 µl of the culture of OD 0.8-1.2 was inoculated into 50 ml of LB + 50 µl carbenicillin medium and incubated on orbital shaker (200 rpm) at 25 °C. When the O.D at 600 nm reached 0.6, the cells were induced with 1mM IPTG and incubated at 25 °C on orbital shaker (200 rpm) for 16 h. After incubation, 20 ml of the culture was centrifuged at 12,000 rpm for 15 min at 4 °C. The pellet was resuspended in 2 ml supernatant and sonicated with protease inhibitor for 20 min under the above mentioned conditions. The supernatant obtained after centrifugation of lysed suspension at 12,000 rpm for 15 min at 4 °C was loaded onto Ni-NTA agarose beads (Qiagen) pre equilibrated with bead wash buffer (50 mM Tris- pH 8.0, 300 mM NaCl, 20 mM imidazole, and 5 % glycerol) and incubated at 4 °C overnight on rocker. After incubation, the bead was washed with wash buffer several times. The elution buffer (50 mM Tris- pH 8.0, 300 mM NaCl, 400 mM imidazole and 5 % glycerol) was loaded onto the beads and

the protein was allowed to elute overnight at 4 °C. The supernatant was used for further analysis. The protein fraction was analyzed for purity by 15 % SDS–PAGE and the protein content of the fraction was estimated by the Lowry's method using bovine serum albumin (BSA) as standard (Lowry et al. 1951). Zymogram analysis of the purified endoglucanase was also performed.

5.4.4.2.C. *Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS PAGE was performed using 4 % stacking gel and 15 % resolving gel. The composition is given in Table 5.7. The samples were mixed with loading dye (Table 5.8) in 3:1 proportion and heated at 95 °C for 10 min. About 20 µl of protein samples were loaded in separate wells and electrophoresis was carried out at 80 V till the dye front crosses the stacking gel and then at 100 V. Current supply was stopped when sample reaches at 0.5 cm above the end of gel. Broad range protein marker (Hi-Range 2, BioLit, SRL) ranging from 14 to 220 kDa was used for determination of molecular weight. The gel was stained with 0.1 % coomassie brilliant blue G-250 prepared in a mixture of methanol: acetic acid: water (40:10:50) and destained with destaining solution. The destaining solution contained methanol, acetic acid and water in same proportion. The bands were visualized and the image was taken.

Table 5.7: Composition of 4 % and 15 % gel

Components	Quantity	
	4 %	**15 %**
ddH_2O	3.4 ml	1.25 ml
40 % acrylamide	830 µl	3.75 ml
1 M Tris buffer (pH 8.8)	-	3.75 ml
1 M Tris buffer (pH 6.8)	630 µl	-
20 % SDS	50 µl	50 µl
10 % APS	50 µl	100 µl
TEMED	5 µl	10 µl
Total volume	5 ml	10 ml

Table 5.8: Composition of SDS loading dye (4X)

Components	Quantity
1 M Tris buffer (pH 6.8)	2 ml
SDS	0.8 g
Glycerol	4 ml
β-mercaptoethanol	3 ml
Bromophenol blue	40 mg
Total volume (made up with ddH_2O)	10 ml

5.4.4.2.D. *Zymogram*

For activity staining of endoglucanase, SDS-PAGE was done using 4 % stacking gel and 15 % resolving gel. The recombinant endoglucanase was mixed with loading buffer (3:1) without β-mercaptoethanol or any reducing agent (SDS) and loaded without heating. After electrophoresis, the gel was immersed in renaturation

buffer (2.5 % triton X-100 in 50 mM sodium citrate buffer, pH 5.0) for 1 h and washed several times to remove SDS. The gel was then laid on to a CMC-agarose plate (1 % CMC and 1 % agarose in 50 mM sodium citrate buffer of pH 5.0). The plate was incubated at 40 °C for 45 min. After this, the gel was removed and the CMC - agarose plate was stained with Congo red (0.1 %) for 30 min followed by destaining with 1 M NaCl till clear zone appeared against a red background (Quiroz-Castañeda et al. 2009).

5.4.5. Characterization of recombinant endoglucanase

In order to characterize the recombinant protein different parameters were studies. The effect of temperature, pH, metal ions, additives, detergents and the substrate specificity of the recombinant endoglucanase was studied. The activities were expressed as relative activity with highest activity as 100 %.

5.4.5.1. *Effect of temperature on activity and stability*

To determine the optimum temperature for endoglucanase activity, the enzyme activity was determined at different temperatures (40 °C - 90 °C). The relative activity at different temperature was examined by keeping the maximum activity as 100 %. To study the thermostability of the enzyme, endoglucanase assay was performed at optimum temperature after incubation of enzyme for 1 h at temperature ranging from 40 °C - 90 °C. The unincubated enzyme was kept as the control and its activity was taken as 100 %.

5.4.5.2. *Effect of pH on activity and stability*

The optimum pH was measured by determining the activity at different pH ranging from 3.0-10.0 using buffers of 50 mM concentration (pH 3.0-6.0, sodium citrate; pH 7.0, sodium phosphate; pH 8.0-10.0, Tris-HCl) at optimum temperature. The maximum activity was taken as 100 %. To study the residual activity the enzyme was incubated with different buffers for 1 h and then assayed under optimum conditions of pH and temperature. The unincubated enzyme was kept as the control and its activity was taken as 100 %.

5.4.5.3. *Effect of metal ions*

The influence of different metal ions (1 mM and 5 mM concentration) on endoglucanase activity was determined by preincubating the enzyme with metal ion solution for 1 h and performing the assay under optimum pH 5.0 and temperature 50 °C. The activity in the absence of metal ion was taken as control (100 %). The metal ions studied were Mn^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, Na^{2+} Fe^{2+}, $Cu^{2+}$and Hg^{2+}.

5.4.5.4. *Effect of additives*

The effect of different additives - Triton X-100, Sodium hypochlorite (SHC), β-mercaptoethanol (β-ME), SDS and EDTA at 0.1 % and 1 % was determined by preincubating the enzyme with the additives for 1 h and then assaying the activity under optimum conditions. The activity in the absence of additives was taken as control.

5.4.5.5. *Effect of commercial detergents*

The effect of different commercially available detergents - Ariel, Surf excel, Tide and Sunlight at 0.1 % and 1 % was determined by preincubating the enzyme with the additives for 1 h and then assaying the activity under optimum conditions. The activity in the absence of additives was taken as control.

5.4.5.6. *Substrate specificity*

The specificity of the recombinant endoglucanase towards different carbohydrate substrate (avicel, cellobiose, filter paper and xylan) was evaluated. All substrates (except filter paper) were examined at 1 % concentration in 50 mM sodium citrate buffer (pH 5.0) with equal volume of enzyme. The mixture was incubated at 50 °C for 30 min. For FPase assay, Whattman filter paper No. 1 (50 mg/ml) was used as substrate and incubated for 1 h under above mentioned conditions. Avicelase, β-glucosidase, FPase activities were measured by the amount of glucose released and xylanase activity was measured by the amount of xylose released per min. CMC was used as the control. All activities were measured relative to endoglucanase activity which was taken as 100 %.

5.4.5.7. *Kinetics of endoglucanase*

The effect of substrate concentration on reaction velocity of purified endoglucanase was determined using different concentrations of CMC. The enzyme activity was assayed at CMC concentration ranging from 2.0 - 22 mg/ml in 50 mM citrate buffer (pH 5.0) at 50 °C

for 30 min. The K_m and V_{max} values were calculated using the software GraphPad Prism 7.04.

5.5. RESULTS

5.5.1. Cloning and transformation of endoglucanase gene into *E.coli* DH5α

5.5.1.1. Genomic DNA extraction

The genomic DNA was extracted and quantitatively and qualitatively analyzed. The concentration of DNA was 1202.9 ng/µl and the ratio of absorbance at 260/280 and 260/230 was 1.99 and 1.89 respectively. On running agarose gel, a single high molecular weight DNA was visible without smear formation.

5.5.1.2. Gradient PCR amplification and standardization

From the range of annealing temperatures studied (51°C – 59 °C), distinct bright bands at position of 1500 bp was obtained at temperature of 51.7 °C and 52.4 °C. At other temperatures, faint bands were obtained at the same position. For all further studies, the annealing temperature was fixed at 52.4 °C (Figure 5.2).

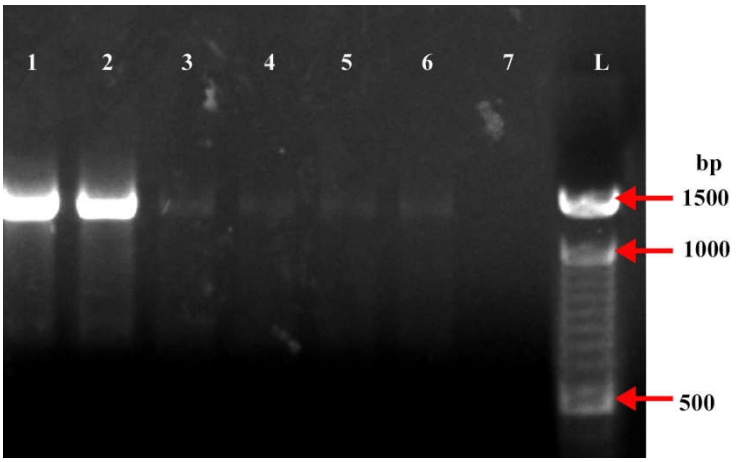
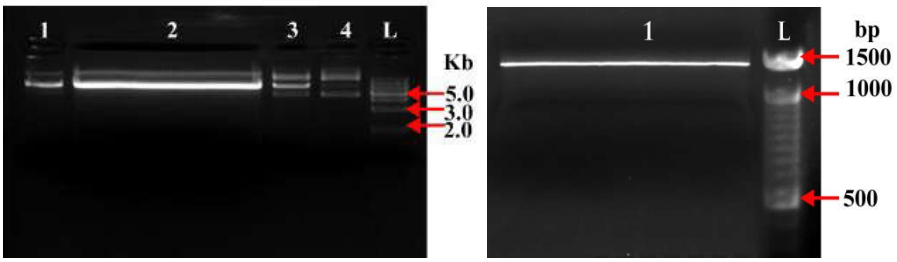


Figure 5.2: Standardization of annealing temperature, lane 1-7: 51.7, 52.4, 53.3, 55.3, 56.2, 57.9, 58.8 °C, lane L- DNA ladder

5.5.1.3. Preparation of insert (PCR product) and vector (plasmid) for ligation

The PCR product was purified using PCR purification kit. After purification, the concentration of the insert was 527.4 ng/µl. The plasmid vector was isolated using QIAGEN® Plasmid Midi Kit. The isolated plasmid had a concentration of 2065.0 ng/µl. Both insert and vector were double digested and run on 1 % gel. A single band was observed in comparison with undigested vector (Figure 5.3). The band was cut from the gel and eluted. The final concentrations of the insert and vector were 64.2 and 76.0 ng/µl respectively. The vector and inset were ligated at low temperature and transformed into *E.coli* DH5α cells.



(A) **(B)**

Figure 5.3: Restriction digestion of **(A)** vector: lane 1- single digest (*Xba* I), Lane 2: double digest, lane 3: single digest (*Xho* I), lane 4: uncut. **(B)** insert: lane 1- double digested insert, lane L: DNA ladder.

5.5.1.4. Ligation and Transformation

Ligation was carried out with two different molar ratios of vector: insert DNA (1:3 and 1:5) and a mixture containing the vector alone without the insert was kept as control. The ligated products were transformed into *E.coli* DH5α cells and spread plated on carbenicillin plates. No colonies were obtained in the control (vector only) plates. The number of colonies in 1:5 plates was higher than in 1:3 plates (Figure 5.4).

Figure 5.4: Transformation plates: **(A)** Colonies in vector only plate **(B)** Colonies in 1:3 plate **(C)** Colonies in 1:5 plate

5.5.1.5. Colony PCR

A total of eight colonies four from each plate (B and C) were randomly selected and colony PCR was performed. Positive clones were observed only in plate C (1:5 plates). Clones C1 and C2 showed bright bands at same position as positive control (1500 bp) confirming transformation of endoglucanase gene (Figure 5.5).

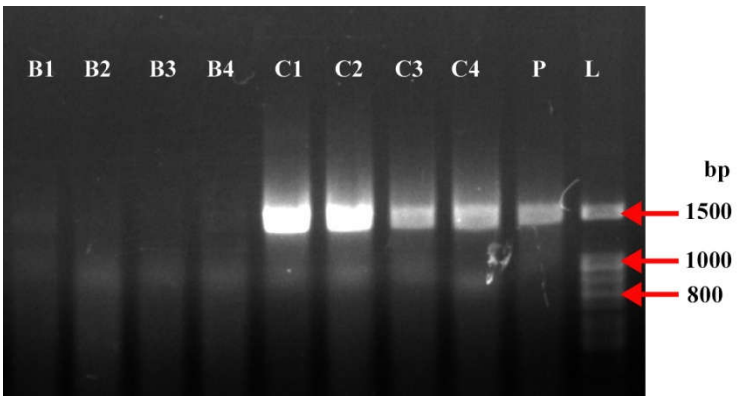
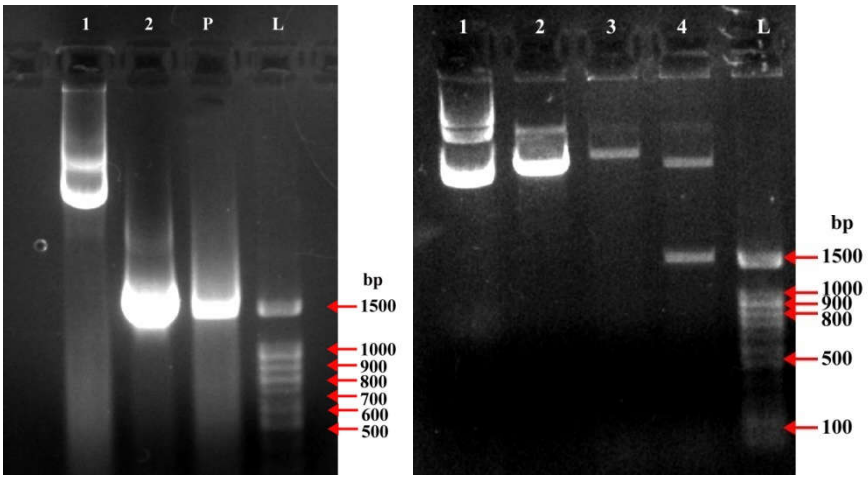


Figure 5.5: Colony PCR: B1-B4- Colonies from 1:3 plates, C1-C4 - Colonies from 1:5 plates, P- positive control, lane L- DNA ladder

5.5.1.6. Plasmid isolation and clone confirmation

Plasmid DNA from clone C1 was isolated and the presence of the endoglucanase gene was confirmed by PCR amplification and restriction digestion of isolated plasmid. Non recombinant plasmid was used as negative control. In recombinant plasmid bright band was observed at same position as positive control. On double digestion of the recombinant plasmid two bands were observed. The band at 1500 bp indicates release of endoglucanase gene from recombinant plasmid, confirming recombination (Figure 5.6).



(A) **(B)**

Figure 5.6: Confirmation of recombinant plasmid. **(A)** PCR amplification- lane 1: non recombinant plasmid, lane 2: recombinant plasmid, lane P: positive control **(B)** Restriction digestion -lane1: uncut vector, lane 2: single cut vector, lane 3: single digested recombinant plasmid, lane 4: double digested recombinant plasmid, lane L: DNA ladder

5.5.2. In-silico characterization of endoglucanase

The endoglucanase gene from recombinant plasmid was sequenced, the vector contamination removed and BLAST analysis was carried out. A sequence of 1462 bp showing 99 % similarity to the endoglucanase of *Bacillus subtilis* subsp. subtilis was obtained (Figure 5.7). The sequence was deposited in Genbank with accession number MG973067.

The sequence analysis of endoglucanase gene revealed an open reading frame (ORF) of 1443 nucleotides encoding a protein of 481 amino acids. The deduced protein had a predicted molecular weight of

53.22 kDa and theoretical pI of 8.14. No signal peptide was predicted from signalP analysis. The enzyme showed a modular structure with a catalytic domain of glycoside hydrolase family 5 (32-278 amino acid) and non – catalytic carbohydrate binding module 3 (332-481 amino acids). The secondary structure analysis showed 61.95 % loop, 15.38 % helix and 22.66 % strand (Figure 5.8).

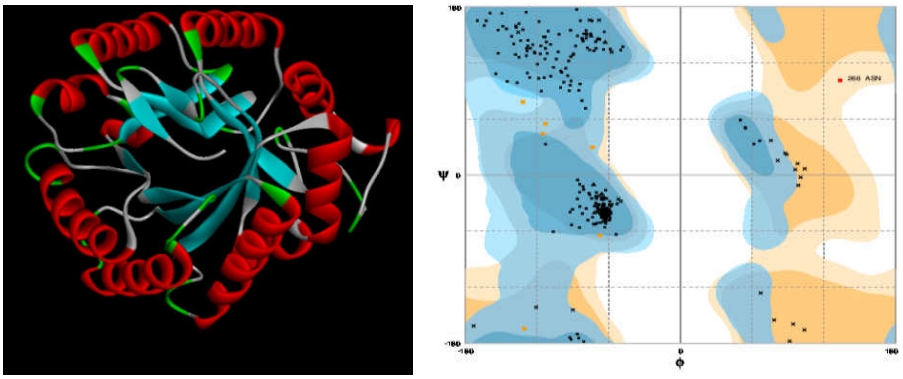
Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus subtilis subsp. subtilis endo-1,4-beta-glucanase (eglS) gene, complete cds	2621	2621	99%	0.0	99%	KP223322.1
Bacillus subtilis strain ge28, complete genome	2580	2580	99%	0.0	99%	CP021903.1
Bacillus sp. YP1, complete genome	2580	2580	99%	0.0	99%	CP010014.1
Bacillus subtilis strain VV2, complete genome	2572	2572	99%	0.0	99%	CP017676.1
Bacillus tequilensis strain S17I28 endoglucanase (cel) gene, complete cds	2572	2572	99%	0.0	99%	KJ854438.1
Bacillus sp. LM 4-2, complete genome	2563	2563	99%	0.0	99%	CP011101.1
Bacillus subtilis strain Ho27 cellulase gene, complete cds	2563	2563	99%	0.0	99%	KF792058.1
Bacillus subtilis subsp. subtilis strain BTN7A endo-1,4-beta-glucanase (egls) gene, complete cds	2563	2563	99%	0.0	99%	KM009052.1
Bacillus subtilis strain shu-3 endoglucanase (eglS) gene, complete cds	2563	2563	99%	0.0	99%	HM470252.1
Bacillus subtilis Y106 alkali tolerable cellulase (cel) gene, complete cds	2563	2563	99%	0.0	99%	AF355629.1
Uncultured bacterium clone celWS20 endo-1,4-beta-glucanase gene, complete cds	2558	2558	99%	0.0	99%	JX567739.1
Bacillus subtilis strain BEC-1 endoglucanase gene, complete cds	2558	2558	99%	0.0	99%	HQ000093.1

Figure 5.7: BLASTn analysis of endoglucanase gene

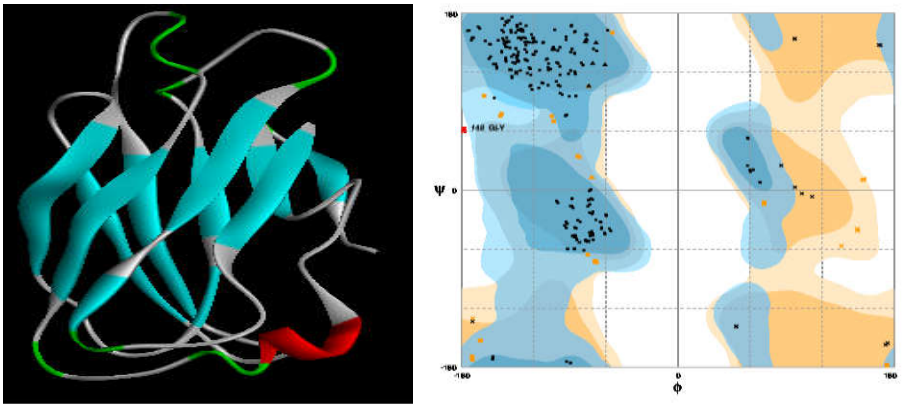
Figure 5.8: Predicted secondary structure of endoglucanase

5.5.2.1. *3D structure prediction by homology modelling*

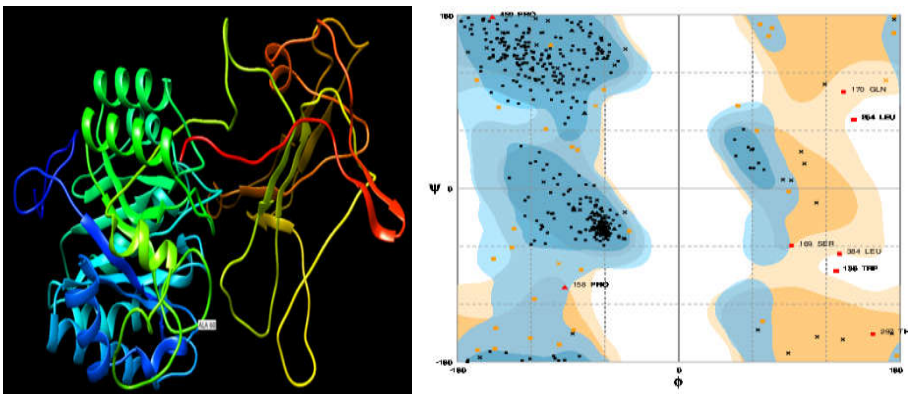
The two domains of endoglucanase were modelled separately and together and presented in Figure 5.9. The catalytic domain showed $(\alpha/\beta)_8$ barrel structure. On superimposition of the modelled structure with the template (Figure 5.10), an RMSD value of 2.04 was obtained.



(A)



(B)



(C)

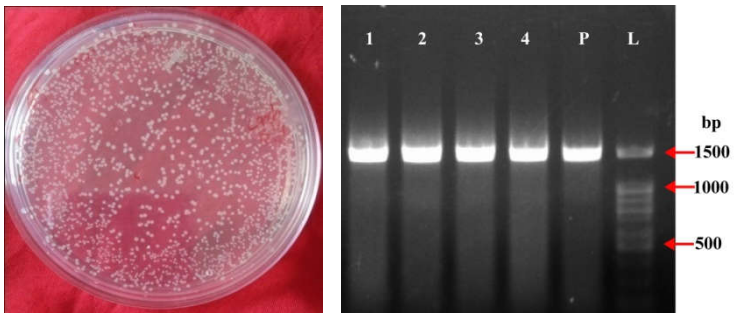
Figure 5.9: Homology modelling of endoglucanase **(A)** catalytic domain **(B)** carbohydrate binding domain **(C)** both the domains together

Figure 5.10: Superimposed structure of endoglucanase. Blue-modelled protein Red –the template (the structural domain of 3PZT and 2L8A)

5.5.3. Transformation and expression of recombinant plasmid in *E.coli* BL-21 (DE3)

5.5.3.1. Sub-cloning into *E.coli* BL-21 (DE3)

The recombinant plasmid was sub-cloned into expression host *E.coli* BL21 (DE3) and positive colonies were selected by colony PCR. On sub-cloning, many colonies (Figure 5.11A) were obtained and all the selected colonies showed incorporation of recombinant plasmid indicated by band at position of 1500 bp (Figure 5.11B).



(A) **(B)**

Figure 5.11: Subcloning of endoglucanase **(A)** Transformed *E.coli* BL-21 (DE3) cells **(B)** Colony PCR: lane 1-4: Amplification of selected colonies, lane P: positive control, lane L: DNA ladder

5.5.3.2. Expression of endoglucanase in *E.coli* BL-21 (DE3)

To study the expression of endoglucanase from *E.coli* BL-21 (DE3), the recombinant colony was grown in LB broth and induced with IPTG. Among the three fractions studied, highest activity was obtained in intracellular fraction suspended in supernatant (279.9 ± 5.2 U/ml), followed by intracellular fraction suspended in citrate buffer (229.99 ± 4.4 U/ml), and finally the cell free supernatant (54.97 ± 3.7 U/ml).

5.5.3.2.A. *Optimization of protein expression*

The expression of endoglucanase was optimized by varying IPTG concentration used for induction. Highest production was obtained when IPTG was used in 1mM (278.84 ± 4.2 U/ml) concentration followed by 0.5 mM (264.1 ± 2.4 U/ml) and 1.5 mM (260.95 ± 4.7 U/ml). On optimization of temperature and incubation time, the highest activity (598.52 ± 5.4 U/ml) was obtained at 25 °C after 16 h of induction (Figure 5.12). The activities obtained were tabulated (Table 5.9). The plate assay of endoglucanase confirms the result (Figure 5.13).

Table 5.9: Effect of incubation time and temperature on endoglucanase production by recombinant *E.coli*

Temperature	**Incubation time (h)**		
	3	**16**	**24**
25 °C	223.84 ± 5.8	**598.52 ± 5.4**	586.19 ± 8.0
30 °C	243.25 ± 9.0	472.61 ± 5.1	465.31 ± 5.0
37 °C	250.2 ± 7.9	278.45 ± 6.7	267.89 ± 5.3

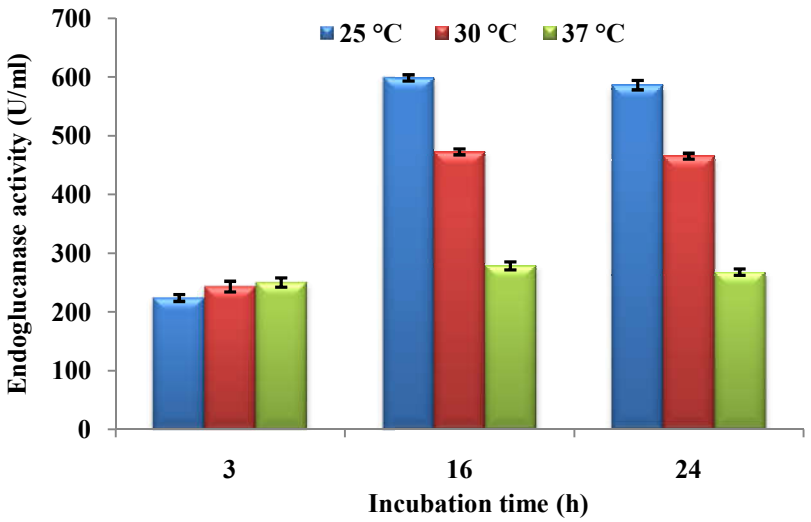


Figure 5.12: Effect of time and temperature on endoglucanase production by recombinant *E.coli*

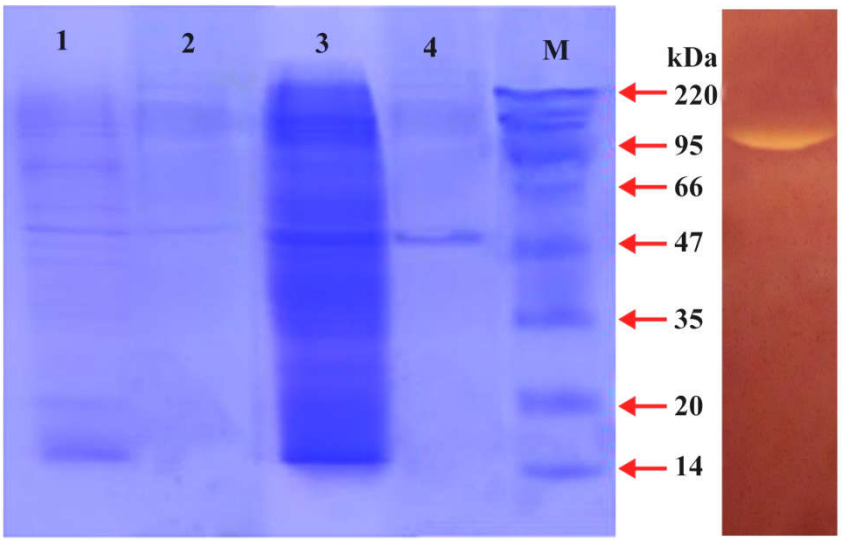
Figure 5.13: Plate assay for endoglucanase **(1)** Positive (supernatant of *Bacillus subtilis* MU S1) **(2)** 3 h recombinant **(3)** 16 h recombinant **(4)** 24 h recombinant

5.5.3.2.B. *Purification of recombinant endoglucanase*

The recombinant protein was purified by affinity chromatography on Ni-NTA agarose beads. The summary of purification is given in Table 5.10. The molecular weight of the expressed endoglucanase was determined by SDS-PAGE. On SDS-PAGE analysis no basal activity was seen in uninduced recombinant *E.coli*. In positive control (*Bacillus subtilis* MU S1) a faint band was observed between 47 and 66 kDa (approximately 53-55 kDa). In protein samples from induced recombinant *E.coli* before and after purification showed a thick band at the same position (Figure 5.14A). On zymogram analysis (Figure 5.14B) of purified endoglucanase, a single band was observed at a position of higher than that obtained in SDS-PAGE.

Table 5.10: Purification of recombinant endoglucanase

Enzyme Preparations	Total activity (U)	Total protein (mg)	Specific Activity (U/mg)	Purification fold	Yield (%)
Recombinant fraction	2992.6	7.9	378.81	1	100
Purified enzyme	1367.84	0.842	1624.51	4.28	45.7



(A) **(B)**

Figure 5.14: SDS-PAGE and zymogram analysis of purified endoglucanase **(A)** lane 1- negative control (uninduced recombinant), lane 2- positive control (supernatant of *Bacillus subtilis* MU S1), lane 3- induced recombinant, lane 4- purified recombinant, lane M- protein marker **(B)** Zymogram analysis of recombinant endoglucanase

5.5.4. Characterization of recombinant endoglucanase

5.5.4.1. *Effect of temperature on activity and stability*

The optimum temperature of recombinant endoglucanase was found to be at 50 °C (Table 5.11). Almost similar activity was observed at 60 °C and this was followed by 40 °C. The activity gradually decreased, retaining 20.68 % activity even at 90 °C. The results of thermostability indicate that endoglucanase retained almost 100 % activity at 40 °C after 1 h incubation. The stability decreased, but the enzyme retained 19.44 % activity even after 1 h incubation at 90 °C (Figure 5.15).

Table 5.11: Effect of temperature on activity and stability

Temperature	Enzyme activity (%)	Enzyme stability (%)
40 ˚C	92.62 ± 1.4	**98.90 ± 1.0**
50 ˚C	**100 ± 0.1**	89.03 ± 0.6
60 ˚C	97.19 ± 1.1	66.54 ± 1.0
70 ˚C	57.75 ± 0.9	45.78 ± 0.3
80 ˚C	32.79 ± 0.8	25.57 ± 1.1
90 ˚C	20.68 ± 1.0	19.44 ± 0.4

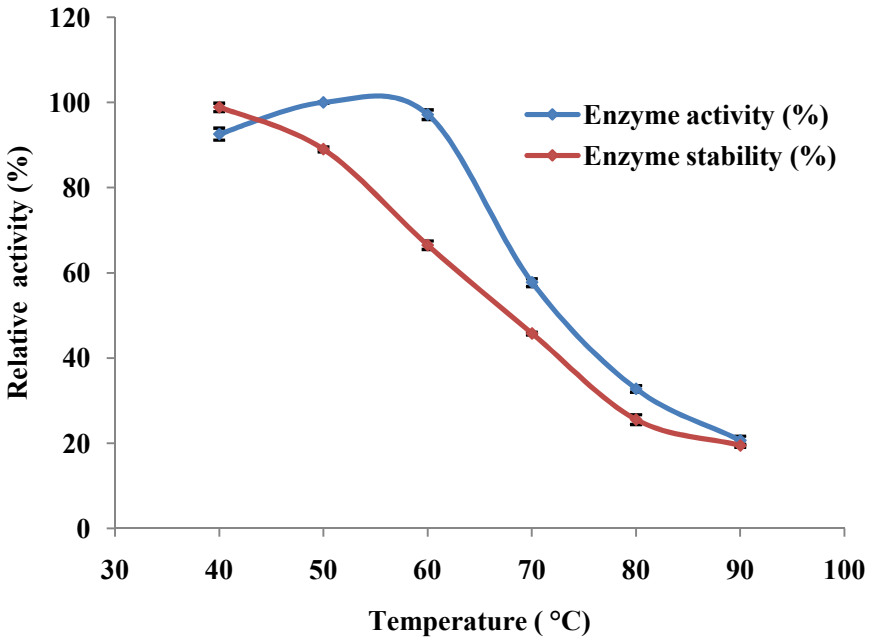


Figure 5.15: Effect of temperature on activity and stability

5.5.4.2. *Effect of pH on activity and stability*

The maximum endoglucanase activity was observed at pH 5.0 (Table 5.12). The endoglucanase activity decreased with increasing pH and retained 44.9 % activity in buffer of pH 10.0. The enzyme was most stable at pH 6.0. The enzyme retained more than 80 % activity in pH range 5.0-10.0 (Figure 5.16).

Table 5.12: Effect of pH on activity and stability

pH	Enzyme activity (%)	Enzyme stability (%)
3.0	33.88 ± 1.3	9.64 ± 1.0
4.0	59.17 ± 0.7	30.57 ± 1.3
5.0	**100 + 0.1**	83.65 ± 0.9
6.0	76.22 ± 0.8	**97.66 ± 1.4**
7.0	65.4 ± 1.2	89.17 ± 1.2
8.0	50.58 ± 1.1	83.50 ± 1.1
9.0	46.06 ± 0.8	82.80 ± 1.3
10.0	44.9 ± 1.5	80.25 ± 0.9

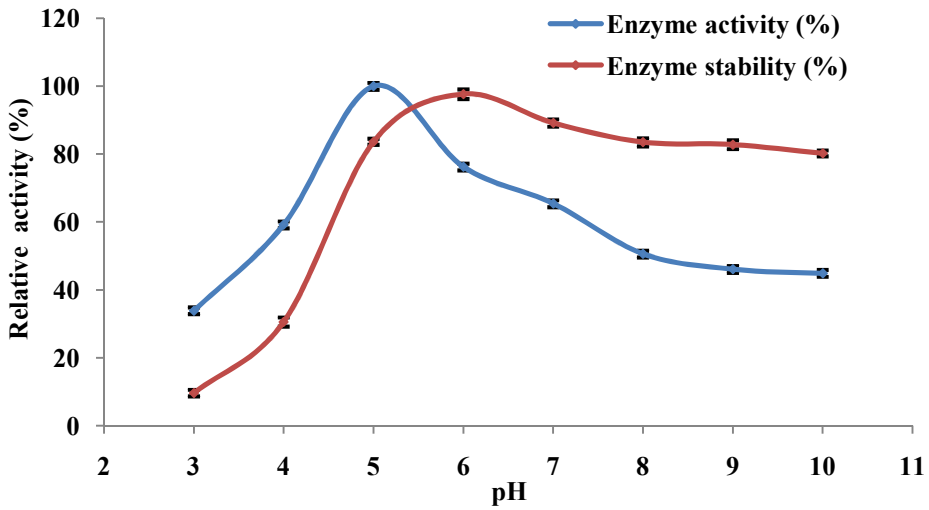


Figure 5.16: Effect of pH on activity and stability

5.5.4.3. *Effect of metal ions*

Among the metal ions tested endoglucanase activity was enhanced by Mg^{2+} and Mn^{2+} at 1 mM and 5 mM (Table 5.13). Fe^{2+} stimulated activity at 1 mM but inhibited activity at 5 mM concentration. All the other metal ions inhibited endoglucanase activity. Hg^{2+} inhibited endoglucanase activity significantly compared to other metal ions (Figure 5.17).

Table 5.13: Effect of metal ions

Metal ions	Relative activity (%)	
	1 mM	5 mM
Control	100 ± 0.6	100 ± 0.4
Mn^{2+}	**104.1 ± 1.6**	**109.56 ± 1.3**
Mg^{2+}	**106.47 ± 1.5**	**112.85 ± 1.7**
Zn^{2+}	81.25 ± 1.0	47.69 ± 1.0
Ca^{2+}	66.99 ± 1.2	99.2 ± 1.6
Na^{2+}	99.02 ± 1.7	90.74 ± 1.4
Fe^{2+}	105 ± 1.1	94.69 ± 0.9
Cu^{2+}	38.28 ± 1.7	31.89 ± 1.2
Hg^{2+}	34.76 ± 1.9	20.73 ± 1.1

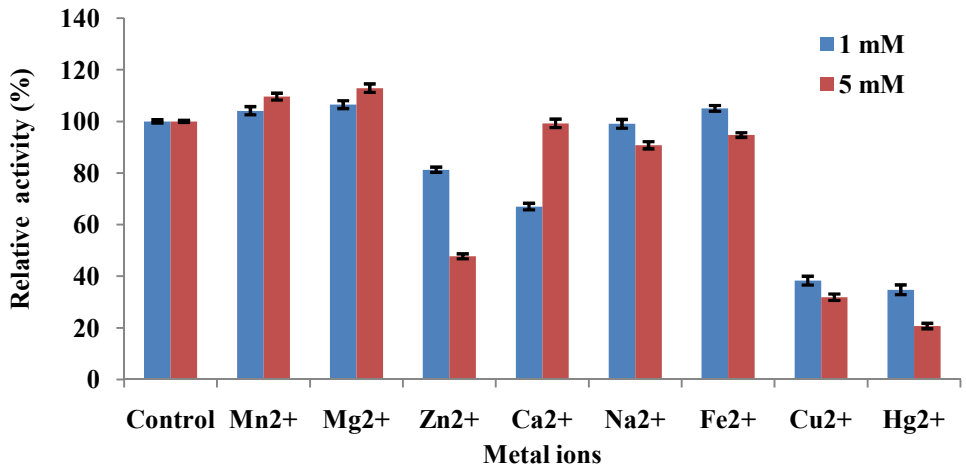


Figure 5.17: Effect of metal ions

5.5.4.4. *Effect of additives*

The effect of different additives on endoglucanase activity showed that 0.1 % SDS (29.77 %) and EDTA (27.64 %) drastically reduced endoglucanase activity (Table 5.14). β-mercaptoethanol retained 79 % and 103 % activity at 0.1 % and 1 % concentration. Sodium hypochlorite decreased endoglucanase activity by less than 50 % at 1 % concentration. Triton X-100 maintained more than 70 % activity at both concentrations (Figure 5.18).

Table 5.14: Effect of additives

Additives	Relative activity (%)	
	0.1%	1%
Control	100 ± 0.5	100 ± 0.6
Triton X-100	74.04 ± 1.2	79.58 ± 2.0
Sodium hypochlorite	69.46 ± 1.6	41.22 ± 1.8
β-mercaptoethanol	79.39 ± 1.8	103.44 ± 1.6
SDS	29.77 ± 1.1	58.78 ± 1.4
EDTA	27.64 ± 1.9	79.77 ± 1.9

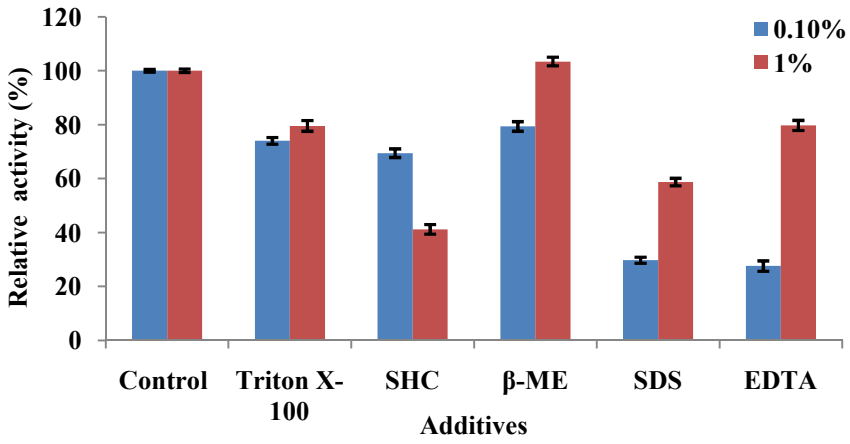


Figure 5.18: Effect of additives

5.5.4.5. *Effect of commercial detergents*

The compatibility of endoglucanase with certain commercial detergents was also determined (Table 5.15). It was observed that the enzyme was stable in the presence of lower concentration of detergents. The activity decreased with increasing concentration of detergent. The enzyme retained 80.7 % activity with 0.1 % and 67.5 % activity at 1 % concentration of Tide. The activity decreased to 17.1 % in the presence of 1 % Ariel (Figure 5.19).

Table 5.15: Effect of commercial detergents

Detergents	Relative activity (%)	
	0.1 %	1%
Control	100 ± 0.6	100 ± 0.8
Ariel	47.54 ± 1.0	17.1 ± 1.1
Surf excel	63.08 ± 1.6	29.28 ± 1.2
Tide	80.7 ± 1.3	67.49 ± 1.6
Sunlight	61.66 ± 1.1	50.9 ± 1.7

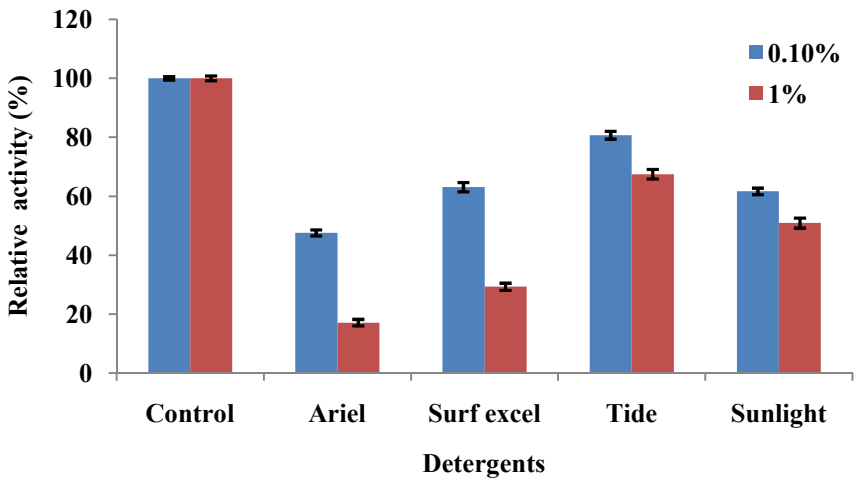


Figure 5.19: Effect of commercial detergents

5.5.4.6. *Substrate specificity*

The substrate specificity of recombinant endoglucanase was studied. The hydrolysis of CMC was kept as control (100 %). The relative activities for the other substrates are presented in Figure 5.20.

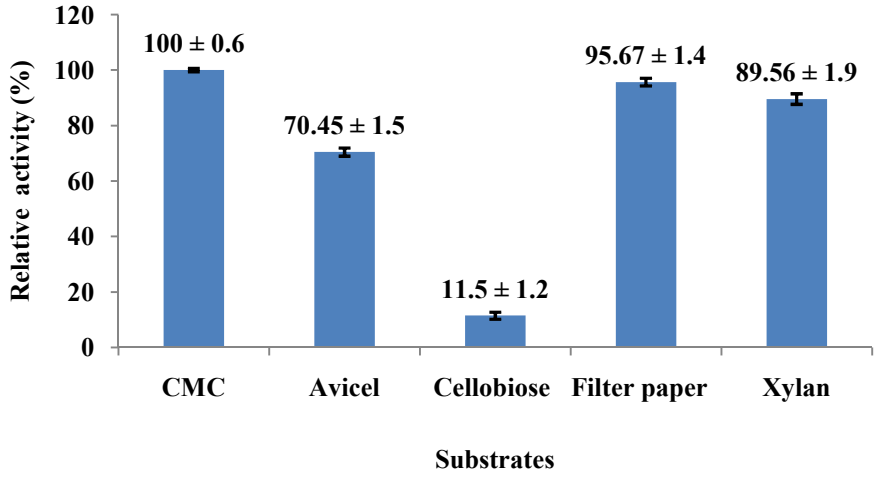


Figure 5.20: Substrate specificity of purified endoglucanase

5.5.4.7. *Kinetics of endoglucanase*

The kinetic parameters of purified endoglucanase were determined by Michaelis-Menten (Figure 5.21) and Line-Weaver Burk (Figure 5.22) plots. The K_m and V_{max} of the enzyme as computed by the software GraphPad Prism 7.04 were 3.97 mg/ml and 905.8 U/ml respectively.

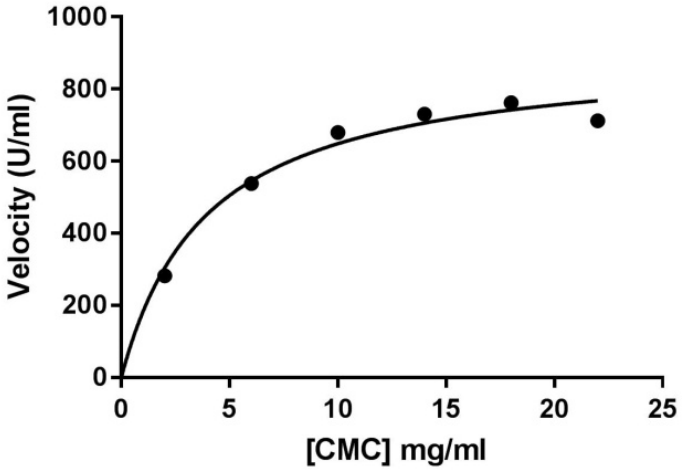


Figure 5.21: Michaelis-Menten plot of purified endoglucanase

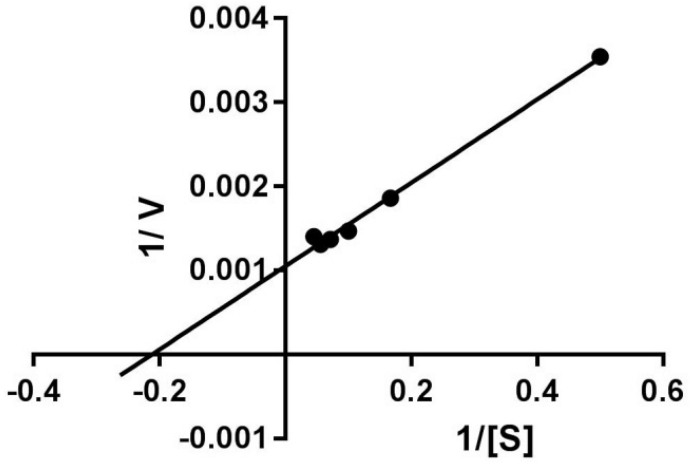


Figure 5.22: Lineweaver–Burk double reciprocal plot of purified endoglucanase

5.6. DISCUSSION

Though *Bacillus subtilis* MU S1 is an efficient endoglucanase producer as reported in chapter 2, the amount of endoglucanase produced was comparatively low (178.54 ± 2.8 U/ ml) for industrial applications. Increased production can be obtained by over-expression of endoglucanase in heterologous hosts. The cloning of endoglucanase from *B.subtilis* in *E.coli,* the most widely used host, has been reported earlier by Pandey et al. (2014), Zafar et al. (2014) and Chuan Wei et al. (2015). In this work, we were successful in cloning the endoglucanase gene from strain MU S1 in pET 22b (+) vector and expressing it in *E.coli* BL-21 (DE3). A sequence of 1462 bp was obtained and on BLASTn analysis the sequence showed 99 % similarity to endoglucanase of *Bacillus subtilis* subsp. subtilis. The sequence was deposited in Genbank with accession number MG973067.

Using bioinformatics tools, the amino acid sequence, secondary and tertiary structures of endoglucanase were predicted. Previous studies on endoglucanase from *Bacillus subtilis* reported an amino acid length of 499. The endoglucanase protein obtained in the present study composed of 481 amino acids and had modular structure consisting of a catalytic and non-catalytic domain. Modular structure of GH5-CBM3 was observed in other *Bacillus subtilis* strains (Santos et al. 2012; Pandey et al. 2014). The difference in the length of protein was due to lack of signal sequence. According to Gopal and Kumar (2013) removal of signal sequence can increase the solubility and expression of recombinant protein. The protein had a predicted molecular weight of 53.22 kDa and theoretical pI of 8.14. The secondary structure

showed high percentage of loops followed by strand and helix. Dhar et al. (2015) also observed a predominance of loops in endoglucanase from *Paenibacillus* sp. IHB B 3084.

On protein expression studies, the endoglucanase activities obtained were in the order: intracellular fraction dissolved in supernatant > intracellular fraction dissolved in buffer > extracellular fraction (supernatant). High endoglucanase activity in intracellular fraction was observed by Chuan Wei et al. (2015), whereas Pandey et al. (2014) and Yang et al. (2010) observed high activity in extracellular fraction. Yang et al. (2010) opined that the signal peptide of endoglucanase in *Bacillus subtilis* strain was responsible for the extracellular localization of the enzyme. In order to obtain higher endoglucanase production, the cultural conditions of recombinant strain were optimized. It was found that highest endoglucanase production was obtained at low IPTG concentration, low temperature and incubation time of 16 h. Liu et al. (2012) also reported high endoglucanase production under similar conditions. Zafar et al. (2014) found that 1 mM IPTG was optimum for endoglucanase production from recombinant *E. coli*. Compared to endoglucanase from *Bacillus subtilis* MU S1 (178.54 U/ml), the maximum activity produced by recombinant endoglucanase from *E. coli* was 598.52 U/ml, which was about 3.4 fold higher than *Bacillus subtilis* MU S1. CMC plate assay which shows a bigger zone of clearance in the recombinants confirms the overexpression of endoglucanase.

The recombinant endoglucanase was purified using Ni-NTA agarose affinity chromatography by a single step. The enzyme was

purified 4.3 folds to homogeneity with 45.7 % yield and a specific activity of 1624.51 U/mg. Cano-Ramirez et al. (2016) reported 4.72 fold purification and 10.58 % yield using Ni-NTA agarose beads. In another study, a purification fold of 9.46 and yield of 22.33 % was reported for recombinant endoglucanase from *Paenibacillus sp*. IHB B 3084 (Dhar et al. 2015). SDS-PAGE analysis was carried out to demonstrate the over-expression and find the molecular weight of the enzyme. The successful over-expression of endoglucanase was confirmed by the presence of thick band in induced recombinant cells compared to uninduced cells. The purified sample gave a single prominent band at ~ 53-55 kDa which was close to the theoretical value (53.22 kDa) calculated using the EXPASYMW tool. A slight higher molecular weight than theoretical may be due to the presence of 6X His tag. Molecular weight between 54-56 kDa was reported for endoglucanase from some *Bacillus subtilis* strains expressed in *E. coli* (Pandey et al. 2014; Zafar et al. 2014; Chuan Wei et al. 2015). Zymogram analysis of recombinant endoglucanase showed a single clear band against red background that confirmed endoglucanase activity. The position of band at approximately 100 kDa indicates that the enzyme acts as a dimer in its native state. McGinnis et al. (1993) also observed dimerization of endoglucanase in unheated sample compared to heated sample.

The optimum temperature of the recombinant endoglucanase was found to be 50 °C. The enzyme was stable over wide range of temperature from 40 °C to 90 °C with highest stability at 40 °C. Same optimum temperature was observed by Zafar et al. (2014) for

recombinant endoglucanase from *Bacillus subtilis* strain. Whereas an optimum of 60 °C and 50 °C - 60 °C was observed by Chuan Wei et al. (2015) and Pandey et al. (2014) respectively. Although the maximum endoglucanase activity was observed at pH 5.0, the enzyme was stable over a wide range of pH. The optimum pH for endoglucanase from different *Bacillus subtilis* strains ranged from near neutral to alkaline pH. Its thermostability and acidophilic property makes this enzyme applicable in biofuel industries.

The endoglucanase activity was stimulated by metal ions like Mg^{2+}, $Mn^{2+}$and Fe^{2+}, slightly inhibited by Zn^{2+}, Ca^{2+}, $Na^{2+}$and strongly inhibited by Cu^{2+} and Hg^{2+}. Zafar et al. (2014) and Padilha et al. (2015) also observed increased endoglucanase activity in the presence of Mg^{2+} and Mn^{2+} respectively. Stabilization of the structure in the presence of the metal ions may be the reason for increased activity. Inhibitory effect of metal ions Cu^{2+}, Zn^{2+}, Hg^{2+} was reported by Chuan Wei et al. (2015) and Yin et al. (2010a). Among all the additives used β-mercaptoethanol at 1 % concentration retained about 100 % endoglucanase activity. Dhar et al. (2015) also reported slight enhancement of activity in the presence of β-mercaptoethanol. Triton X-100 at both concentrations showed low inhibition of activity, whereas, SDS and EDTA considerably decreased the endoglucanase activity. Inhibitory effect of the two additives on endoglucanase activity was observed by Zafar et al. (2014) and Yin et al. (2010a). Sodium hypochlorite showed a moderate effect on enzyme inhibition.

Endoglucanase has wide application in detergent industries; therefore, the compatibility of the recombinant endoglucanase with

some of the commercially available detergents was monitored. The enzyme was highly stable in the presence of 0.1 % concentration of detergent Tide. In all cases at higher concentration of detergents the activity diminished. Similar observations were made by Sadhu et al. (2013). The stability of enzyme in high pH range may be the reason for its stability in the presence of detergents. The specificity of recombinant enzyme was tested by its ability to hydrolyze different substrates. The recombinant endoglucanase showed highest activity towards CMC confirming that the expressed enzyme was endoglucanase. The enzyme hydrolyzed the other substrates in the order filter paper > xylan > avicel > cellobiose. The catalytic domain of endoglucanase belongs to GH5 and this may be the reason for multiple enzyme activity. Chuan Wei et al. (2015) observed activity of cloned endoglucanase towards CMC, cotton linter, avicel and filter paper, but no activity was seen towards xylan and 4-nitrophenyl α-D glucopyranoside.

Kinetic parameters are important factors that determine the catalytic efficiency of an enzyme. The K_m measures the affinity of the enzyme towards the substrate. Lower the value of K_m, higher is the affinity. V_{max} represents maximum velocity of a reaction, that is, how fast the enzyme can catalyze the reaction. The K_m and V_{max} values of recombinant endoglucanase were found to be 3.97 mg/ml and 905.8 U/ml respectively. Low K_m indicates high affinity of endoglucanase towards CMC, whereas high V_{max} indicates its high rate of catalysis. Yang et al. (2010) reported a K_m of 3.59 mg/ml for recombinant endoglucanase from *Bacillus subtilis*. Rawat and Tewari (2012)

observed a K_m value of 2.2 mg/ml and V_{max} of 699.0 U/ml for endoglucanase from *Bacillus subtilis* strain LFS3. Gaur and Tiwari (2015) reported K_m and V_{max} values 1.923 mg/ml and 769.230 µg/ml/min, respectively for endoglucanase from *Bacillus vallismortis* RG-07. Zafar et al. (2014) recorded a K_m - 1.76 µmol and V_{max} - 0.20 µmol/min for a recombinant endoglucanase from *Bacillus subtilis* expressed in *E.coli.*

5.7. CONCLUSION

In this chapter, we report the successful cloning and expression of endoglucanase gene from *B.subtilis* MU S1 in *E. coli*. The recombinant endoglucanase showed a 3.4 fold increase in endoglucanase activity as compared to strain MU S1. Characterization of the endoglucanase indicated that it was an acido-thermophilic enzyme showing stability over a wide range of temperature and pH. The stability of endoglucanase in the presence of detergents and its ability to degrade different substrates increases its applicability in different industries, especially in detergent and biofuel industries.

SREENA C. P. “IMPROVEMENT OF BIOCATALYTIC EFFICACY OF ENDOGLUCANASE FROM INDIGENOUS STRAIN, BACILLUS SUBTILIS MU S1 AND ITS APPLICATION IN SACCHARIFICATION OF AGRO-WASTES”. THESIS. DEPARTMENT OF LIFE SCIENCES, UNIVERSITY OF CALICUT, 2018.

6.1. INTRODUCTION

The limited supply and high cost of fossil fuels, in addition, the environmental pollution caused by its excessive usage has forced the world economy to focus on biofuels especially bioethanol from renewable resources. First generation bioethanol is obtained from food crops and it is considered to be economical and environment-friendly. But the major disadvantage with the widespread use of these fuels is the increase in the cost of food crops which forms the bases of food-versus-fuel debate. Second generation bioethanol circumvents these issues by making use of cheap and abundant nonfood lignocellulosic biomass residues.

The conversion of lignocellulosic biomass into bioethanol involves following steps: pretreatment, hydrolysis (saccharification) and ethanol recovery. Among these, hydrolysis of biomass to fermentable sugars is the essential step. These sugars are later converted to alcohol by a fermentative organism. Hydrolysis can be achieved by two methods: acid and enzymatic hydrolysis. Acid hydrolysis has the disadvantage of formation of hazardous acidic wastes which makes recovery of sugars difficult. The enzymatic hydrolysis process does not generate any toxic waste, is more efficient and proceeds under ambient conditions (Mishima et al. 2006). Cellulases and hemicellulases are the key enzymes involved in complete saccharification of the substrate. Thus the most important application of cellulase which is currently being investigated is in bioconversion of lignocellulosic wastes into biofuels. But the major issues related to enzymatic saccharification is the cost of enzymes, the

high incubation time for saccharification, degradation of enzymes in the presence of phenolic compounds and reaction temperature (Madadi et al. 2017). Improving the technologies for saccharification by use of efficient enzyme cocktails and hydrolysis conditions can bring down the cost of biofuel production.

Different agro-wastes like jamun (*Syzygium cumini*), neem (*Azadirachta indica*), asoka (*Saracens indica*), bamboo (*bambusa dendrocalmus*), poplar (*Populas nigra*), wild grass (*Achnatherum hymenoides*), eucalyptus (*Eucalyptus marginata*), mango (*Mangifera indica*) (Mutreja et al. 2011), wheat straw, bagasse (Haq et al. 2015), bamboo biomass (Ali et al. 2015), wheat bran, corn cobs, sunflower stalks, rice straw, and rice bran (Phadtare et al. 2017) have been used as substrates for saccharification by recombinant endoglucanases. Pepper, arecanut, tamarind, banana and jackfruit are amongst the important crops cultivated in Kerala. To the best of our knowledge, there are no reports on the use of recombinant endoglucanase for saccharification of these agro-wastes.

6.2. OBJECTIVES

The main aim of this chapter was to investigate the saccharification potential of recombinant endoglucanase using few locally available agro-wastes. The process parameters for efficient saccharification of selected agro-waste were optimized. Finally, hydrolysis was tried using enzyme preparations.

6.3. REVIEW OF LITERATURE

The declining supply of fossil fuels has encouraged researchers to look for renewable resources as an alternative. Biofuel research mainly includes products like bioalcohol (mainly bioethanol), biodiesel, biohydrogen and biogas. Bioethanol is the most promising renewable energy for replacement of fossil fuel, because of its properties like, greater air-fuel ratio, reduction of CO_2 emission, high energy density and more heat of vaporization (Dias De Oliveira et al. 2005). There are mainly two approaches for bioethanol production: direct and indirect fermentation. Direct fermentation involves the use of plant material for ethanol production, first by saccharification of plant material and then by conversion of the sugars generated to ethanol. Indirect method involves pyrolysis of the plant material followed by conversion of produced gas to ethanol by acetogenic bacteria (Klasson et al. 1992).

The first and most important step for bioethanol production by direct fermentation is saccharification of plant material. Depending on the starting material to be converted to sugars, bioethanol can be divided into first and second generation. First generation bioethanol uses sugar and starch containing feedstocks that are easily degraded; whereas second generation bioethanol is obtained from cheap lignocellulosic materials, but are difficult to degrade. Sugar crops like sugarcane, sugar beet (*Beta vulgaris*), watermelon (*Citrullus lanatus*), sweet sorghum, dates (*Phoenix dactylifera*) have been used as sugar juices for bioethanol production (Zabed et al. 2014). Starch-based feedstocks used for alcohol production are barley (*Hordeum vulgare*),

corn, yam (*Dioscorea alata*), sweet potato (*Ipomoea batatas*), potato, cassava (*Manihot esculenta*), and grains like wheat and maize (Thatoi et al. 2014).

As compared to sugar and starch feedstocks, lignocellulosic biomass has the advantage of being cheap and promising non-food raw material which can be utilized without depleting reserves. However, the structural features of biomass make the conversion process difficult. Various pretreatment methods are available depending on the application and type of substrate. These have been discussed in chapter 4. The pretreatment methods disrupt the lignin-cellulose complex and make cellulose available for hydrolysis. Saccharification during hydrolysis is the most important and rate-limiting process in the techno-economic feasibility of second generation bioethanol (Madadi et al. 2017). As compared to acid hydrolysis, enzymatic hydrolysis is preferred as it is carried out at mild conditions (usually pH 4.8 and temperature 45-50 ^{o}C), does not have any corrosion problem nor generate any inhibitory compounds. Also, hydrolysis up to 100 % can be obtained by use of enzymes (Duff and Murray 1996).

Cellulases and hemicellulases (mainly xylanase) are the main enzymes involved in saccharification of lignocellulosic biomasses. These enzymes act on cellulosic and hemicellulosic components of lignocellulosic material resulting in the formation of hexoses (glucose) and pentoses (xylose), respectively. These sugars (mainly glucose) are then fermented to ethanol by yeast *Saccharomyces cerevisiae*. Over past few years, Gram-negative bacteria like *Zymomonas mobilis, Escherichia coli* and *Klebsiella oxytoca* have been engineered to

ferment sugars (mainly xylose) which are not fermented by yeast (Dien et al. 2003).

6.3.1. Saccharification of lignocellulosic wastes

Saccharification of different agro-wastes has been reported by researchers using cellulases from different organisms. Fungi have been widely exploited as a source of cellulases for saccharification. Different agro-wastes used for saccharification by fungal cellulases are palm cake and palm fibre, Kallar grass (*Leptochloa fusca*) straw, eucalyptus, douglas fir (*Pseudotsuga menziesii*), corncobs, sugarcane bagasse, rice straw, sorghum straw, dried flower, coconut shell, water hyacinth (*Eichhornia crassipes*), orange (*Citrus sinensis*) peel, sawdust, pearl millet (*Pennisetum glaucum*) straw, rinds of pineapple (*Ananas comosus*), jackfruit, watermelon and muskmelon (*Cucumis melo*), pumpkin (*Cucurbita*) residues, dried leaves and psuedostem of banana, wheat straw, rice bran, mesquite (*Prosopis juliflora*), and groundnut shells (Karmakar and Ray 2011b; Begum and Alimon 2011; Bhandari et al. 2013; Sridevi et al. 2015).

Although bacterial cellulases are more complex and provide all the enzymes of cellulase system they are less exploited for saccharification studies. Premalatha et al. (2015) used cellulase from *Enhydrobacter* sp. ACCA2 for saccharification of sorghum leaf, sorghum stem, bamboo, cumbu (pearl millet) leaf, and cumbu stem. Among this maximum saccharification was observed in bamboo. Singh et al. (2015) studied saccharification of Santa Maria feverfew (*Parthenium hysterophorus*). Sheng et al. (2016) used rice straw, corn

stalks, corncob, and poplar sawdust for saccharification using a new thermophilic bacterium, *Ruminiclostridium thermocellum* M3. Alrumman (2016) used date palm (*Phoenix dactylifera*) wastes as a substrate for hydrolysis using *Geobacillus stearothermophilus.* The efficiency of endoglucanase from *Bacillus subtilis* K-18 for hydrolysis of pine (*Pinus*) needles was studied by Irfan et al. (2017).

Recently, recombinant cellulases have been used for saccharification studies. Szijarto et al. (2011) found that heterologously expressed endoglucanase was more efficient in reducing the viscosity of biomass as compared to other heterologous enzymes tested (CBHs, xylanases and β-glucosidase). Mutreja et al. (2011) used recombinant cellulase from *Clostridium thermocellum* for saccharification of jamun, eucalyptus, neem, mango, asoka, bamboo, wild grass, and poplar. Haq et al. (2015) used recombinant endoglucanase from another strain of *Clostridium thermocellum* for saccharification of pretreated wheat straw and bagasse. An endo-1,4-β-glucanase gene, of *Thermotoga petrophila* RKU-1, was cloned and over-expressed in *E. coli* strain BL21 and was used for saccharification of wheat straw and bagasse by Ul Haq et al. (2015) . Ali et al. (2015) cloned three genes: GH3 family β-glucosidase (BGL), GH7 family-related cellobiohydrolases (CBHs), and endoglucanase (EG) from a newly isolated *Aspergillus niger* BE-2 and expressed in *Pichia pastoris* GS115. The obtained enzyme was used for hydrolysis of bamboo biomass. Phadtare et al. (2017) tried saccharification of wheat bran, corncobs, sunflower stalks, rice straw, and rice bran using recombinant thermo-alkali-stable endoglucanase of *Myceliopthora thermophila* BJA.

6.3.2. Factors affecting enzymatic saccharification of lignocelluloses

Enzymatic saccharification of lignocelluloses is affected mainly by substrate and enzyme related factors and their interactions. The structural features of substrate affecting hydrolysis are lignin and hemicellulose content, cellulose crystallinity, the degree of cellulose polymerization, particle size and surface area accessible to cellulase (Gregg and Saddler 1996). Lignin and hemicellulose content of the substrate plays important role in determining the susceptibility of the substrate to hydrolysis, as they are connected to cellulose by covalent and non-covalent linkages. As lignin and hemicellulose content increases the hydrolysis rate decreases. Moreover, cellulase gets irreversibly adsorbed to lignin thus making it unavailable for further hydrolysis of the substrate (Pan et al. 2005).

The crystallinity of cellulose (Crystallinity Index, Crl) is another factor that determines the hydrolysis rate. In a highly crystalline substrate, the hydrogen bond is closely packed than in amorphous regions, hence less accessible to the enzyme. During hydrolysis amorphous region is digested before the crystalline region. Pretreatments that reduce cellulose crystallinity and lignin content can increase biomass digestibility. The degree of polymerization (DP) is highly variable among different plant species and is related to the crystallinity of the structure. Decreased DP in substrate prepared by some pretreatment procedures suggested that xylan reduction had a greater effect on chain length reduction than the removal of lignin. Particle size and accessible surface area are very important parameters that influence enzymatic hydrolysis. Decreasing the particle structure

opens the tightly packed cell wall and allows easy access of enzymes to the carbohydrate. The cellulose hydrolysis occurs faster as more cellulose is exposed to the enzymes (Madadi et al. 2017).

The enzyme related factors that affect saccharification are cellulase activity, cost of the enzyme, end product inhibition, thermal stability, and composition of the cocktail. Cellulase enzyme suffers end product inhibition by glucose and cellobiose. The enzyme activity is affected more by cellobiose accumulation compared to glucose accumulation showing that cellobiose is a stronger inhibitor (Holtzapple et al. 1990). End product inhibition can be overcome by using a high concentration of enzymes, supplementation of cellulases with exogenous cellobiase (β-glucosidase) activity, elimination of sugars from hydrolysate by ultrafiltration or by simultaneous saccharification and fermentation of the substrate (Gregg and Saddler 1996). A high temperature is preferred for saccharification, as it disrupts the crystalline structure by destabilization of H bonds and thus increases the degree of saccharification (Haq et al. 2015). Therefore thermal stability of the enzyme is an important factor. For hydrolysis reaction, highly efficient enzymes with high specific activity are desirable so as to obtain high saccharification rates at low concentration of enzyme.

As complete enzymatic hydrolysis of biomass involves the concerted action of all the enzymes of cellulase system (endoglucanase, exoglucanase and β-glucosidase), hemicellulases and other accessory enzymes, the composition of enzyme cocktail is a major factor that affects not only the cost but also the efficiency of

hydrolysis. Recent studies focus on enzyme engineering and designing of enzyme cocktails for complete hydrolysis. Engineering multifunctional enzymes having more than one catalytic site and a cellulose binding module can increase enzymatic hydrolysis by concerted action on the cellulosic substrate. There are some endoglucanases with both endo and exo activities. These are called processive endoglucanases. Therefore instead of using separate endo and exoglucanase, use of processive endogolucanase with cellobiase could be cost effective. Most of the known processive endoglucanase belong to GH9 and have a modular structure with catalytic domain (GH9) and CBM. Haq et al. (2015) used processive endoglucanase of GH9 family for saccharification of pretreated wheat straw. Very few processive enzymes from family GH5 have been identified till date. Wu et al. (2018) identified a processive endoglucanase from *Bacillus subtilis* BS-5 with application in lignocellulosic saccharification. Finally, the interaction between the type of substrate and the enzyme is very important. The overall saccharification rate can be enhanced by optimizing the various reaction parameters like concentration of substrate, enzyme and reaction conditions (temperature, pH).

6.4. MATERIALS AND METHODS

6.4.1. Enzyme source

Recombinant endoglucanase with an activity of 598.52 U/ml was used for saccharification studies.

6.4.2. Collection and pre-treatment of agro-wastes

The same agro-wastes used in chapter 4 were used for saccharification studies (pepper waste, banana peduncle, arecanut husk, tamarind husk and jackfruit outer rind). All the wastes were treated in the same way as described in section 4.4.2 of chapter 4.

6.4.3. Enzymatic saccharification of agro-wastes

Enzymatic hydrolysis of agro-wastes was tried with recombinant endoglucanase. Saccharification was performed in 100 ml Erlenmeyer flask with 1 % steam exploded agro-wastes in 10 ml of 50 mM sodium citrate buffer (pH 5.0). The flasks were then autoclaved for 20 min at 121 ^{o}C to prevent microbial contamination (Sridevi et al. 2015). To these flasks, 0.5 ml (30 U) of the enzyme was added and incubated at 50 ^{o}C for 5 days. Flasks without enzyme served as control. Samples were withdrawn after every 24 h, the contents were centrifuged at 10,000 g for 10 min and the sugar content in the supernatant was analyzed using DNS method (Miller 1959). All experiments were carried out in duplicates. The percentage of saccharification (Spano et al. 1976) was calculated using the following formula

$$\textbf{Saccharification (\%)} = \frac{\textbf{Reducing sugars (mg/ml)} \times 0.9 \times 100}{\textbf{Initial substrate concentration (mg/ml)}}$$

The multiplication factor 0.90 was employed to convert polysaccharide to monosaccharide and accounts for the uptake of water during hydrolysis.

6.4.4. Optimization of saccharification

The different parameters that affect saccharification percentage of the selected agro-waste (pretreated JOR) were optimized. The parameter studied were temperature, pH of the buffer, the concentration of substrate and enzyme. The optimum condition obtained for each experiment was used in next optimization.

6.4.4.1. *Effect of temperature*

The effect of temperature was studied by incubating the saccharification set up at three different temperatures (40 ^{o}C, 50 ^{o}C and 60 ^{o}C) for 5 days. A control was kept at each temperature.

6.4.4.2. *Effect of pH*

The optimum pH for saccharification of JOR was studied by varying the reaction buffer pH from 4.0 -7.0 using buffers of 50 mM concentration (pH 4.0-6.0, sodium citrate; pH 7.0, sodium phosphate). The experimental set up was incubated for 5 days at 50 ^{o}C. A control was kept for each buffer.

6.4.4.3. *Effect of substrate concentration*

In order to study the optimum substrate concentration saccharification was carried out with different concentrations of JOR (5, 10, 15, 20, and 25 mg/ml) in a buffer of pH 5.0. The experimental set up was incubated for 5 days at 50 ^{o}C. A control was kept for each substrate concentration.

6.4.4.4. *Effect of enzyme concentration*

The optimum concentration of the enzyme required to hydrolyze JOR was determined by varying the concentration of enzyme used for saccharification (6, 18, 30, 42, and 54 U), keeping all other factors at its optimized conditions. In control flasks, different concentrations of heat-inactivated enzyme were added.

6.4.5. Saccharification using different enzyme preparations

Saccharification was carried out using different enzyme preparations under the optimized set of hydrolysis conditions. The different enzyme preparations used were (i) crude enzyme obtained from *B. subtilis* MU S1 in unoptimized medium (E1-178.54 U/ml), (ii) crude enzyme obtained from *B.subtilis* MU S1 in optimized medium (E2-566.62 U/ml), (iii) recombinant endoglucanase (E3-598.52 U/ml), (iv) cocktail (CT) 1 (equal concentrations of E1 and E3), (v) cocktail 2 (equal concentrations of E2 and E3). For saccharification the flasks containing 15 mg/ml of pretreated JOR in 10 ml sodium citrate buffer (pH 5.0) was sterilized, loaded with 18 U of different enzymes and incubated at 50 ^{o}C for 5 days. In control flasks, the same concentration of heat inactivated enzymes was added.

6.5. RESULTS

6.5.1. Enzymatic saccharification of agro-wastes

Among the various agro-wastes tested for hydrolysis, the highest saccharification was observed in case of JOR (33.4 %), followed by BP (23.1 %), PW (22 %), TH (21 %) and AH (14.9 %). In

all cases highest saccharification was observed at 96 h of incubation. No significant increase was seen after further incubation. As the maximum saccharification was observed for JOR, it was used for further studies.

Table 6.1: Screening of agro-wastes

	Saccharification (%)					
Agro-wastes	**0 h**	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**
JOR	0	18	20.7	26.4	**33.4**	33
BP	0	11.1	18.2	20.1	**23.1**	21.2
TH	0	7.85	13.3	15.1	20.7	**21**
AH	0	3.65	5.15	6.3	14.1	**14.9**
PW	0	2.20	4.95	17.8	21.7	**22**

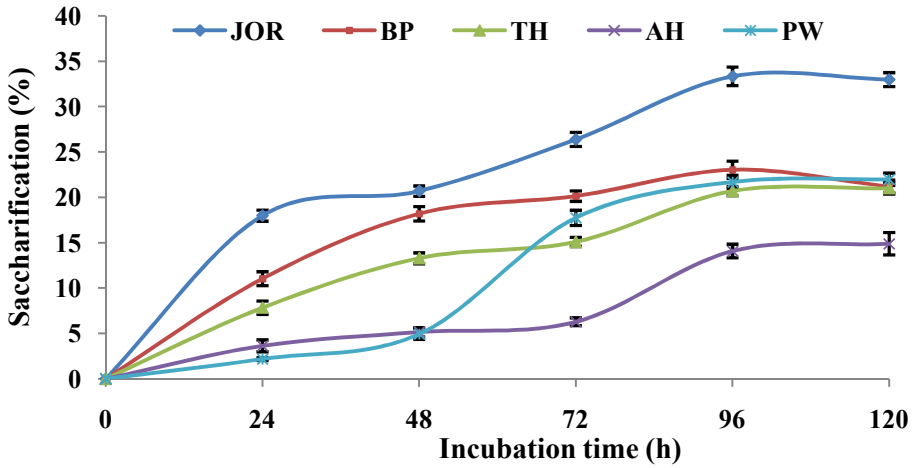


Figure 6.1: Screening of agro-wastes

6.5.2. Optimization of saccharification of JOR

The different parameters controlling the saccharification rate was optimized by OFAT method.

6.5.2.1. *Effect of temperature*

The optimum temperature for saccharification of JOR was 50 °C (33.4 %) followed by 40 °C and 60 °C.

Table 6.2: Effect of temperature on saccharification of JOR

	Saccharification (%)					
Temperature	**0 h**	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**
40 °C	0	9.3	14.9	21.1	29.2	28.9
50 °C	0	12.9	20.1	29.8	**33.4**	33.0
60 °C	0	5.3	12.4	18.4	22.8	20.9

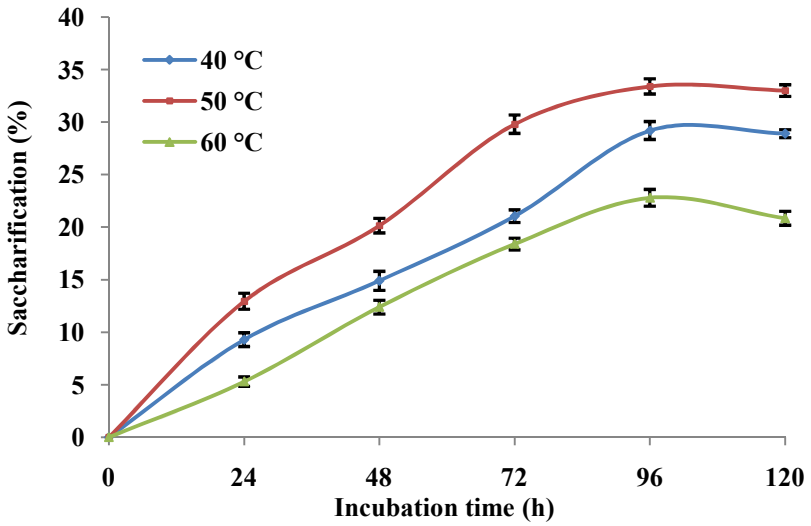


Figure 6.2: Effect of temperature on saccharification of JOR

6.5.2.2. *Effect of pH*

The optimum pH for saccharification of JOR was pH 5.0 (33.9 %). Almost similar saccharification percentage was observed at pH 6.0, followed by pH 7.0. The hydrolysis decreased at pH 4.0.

Table 6.3: Effect of pH on saccharification of JOR

	Saccharification (%)					
pH	**0**	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**
4.0	0	7.8	14.4	19.2	23.9	22
5.0	0	11.8	18.2	28.6	**33.9**	33.5
6.0	0	18.4	21.2	26.9	32.6	32.1
7.0	0	5.4	14.3	23.3	29.6	29.1

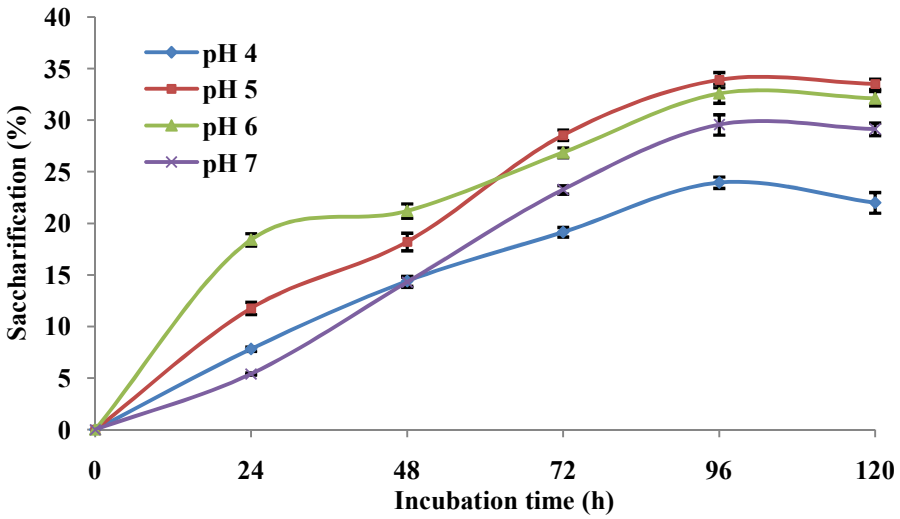


Figure 6.3: Effect of pH on saccharification of JOR

6.5.2.3. *Effect of substrate concentration*

The optimum concentration of the substrate for hydrolysis of JOR was 15 mg/ml (37.8 %), while at other concentrations saccharification decreased. The hydrolysis declined considerably at high substrate concentration of 25 mg/ml (7.86 %).

Table 6.4: Effect of substrate concentration on saccharification of JOR

Substrate conc. (mg/ml)	Saccharification (%)					
	0 h	24 h	48 h	72 h	96 h	120 h
5	0	2.1	4.55	12.3	20.2	19.6
10	0	14.2	19.2	29.9	34	33.8
15	0	19.9	22.1	29.1	**37.8**	36.8
20	0	8.1	16.65	20.8	24.3	23.9
25	0	4	6.35	6.8	7.15	7.86

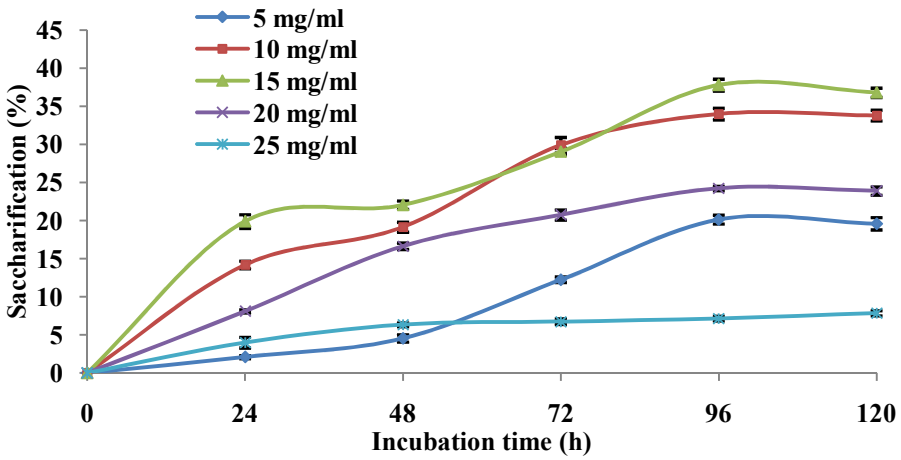


Figure 6.4: Effect of substrate concentration on saccharification of JOR

6.5.2.4. *Effect of enzyme concentration*

The maximum sugar production was recorded on loading 18 U (41.1 %) of recombinant endoglucanase.

Table 6.5: Effect of enzyme concentration on saccharification of JOR

Enzyme conc. (U)	Saccharification (%)					
	0	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**
6	0	12.8	23.2	26	31	29.2
18	0	17.8	23.9	308	**41.1**	40.5
30	0	16.7	20.1	29.1	38	37.5
42	0	10.3	15.7	18.9	27	26.5
54	0	10.7	12.9	15.5	18.9	16.7

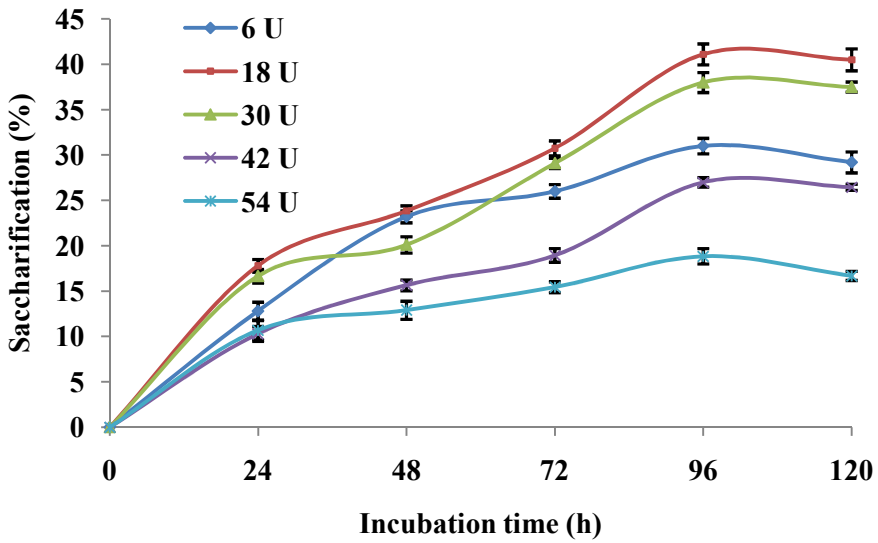


Figure 6.5: Effect of enzyme concentration on saccharification of JOR

6.5.3. Saccharification using different enzyme preparations

Among the different enzymes, CT 1 (42.5 %) and E3 (41 %), gave the highest saccharification rate.

Table 6.6: Saccharification of JOR using enzyme preparations

	Saccharification (%)					
Enzymes	**0 h**	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**
E1	0	3.3	6.7	11.5	15.8	16
E2	0	4.3	12.3	16.4	24.5	24.6
E3	0	16.6	24.4	30.1	41	40.8
CT 1	0	20.9	26.3	34.2	**42.5**	42.1
CT 2	0	14.8	18.5	21.4	31.7	30.1

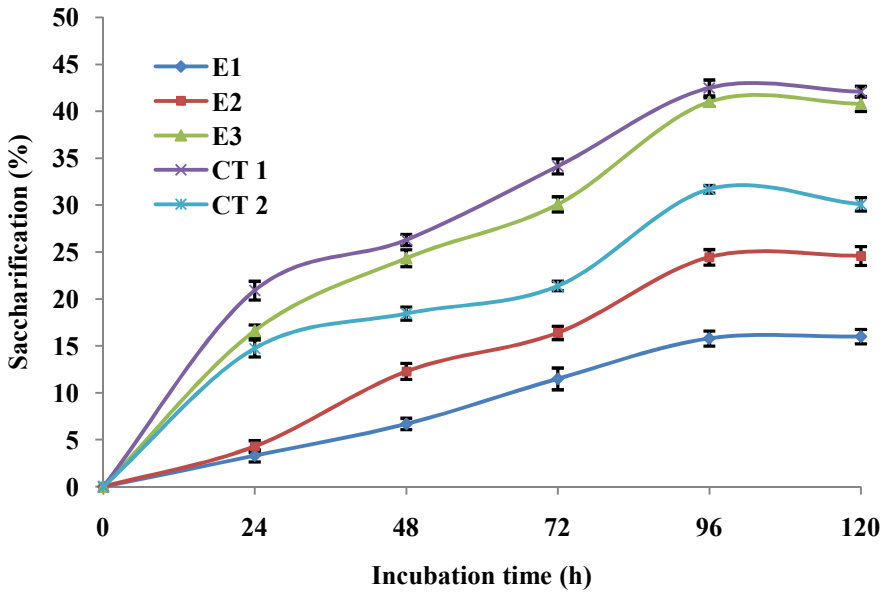


Figure 6.6: Saccharification of JOR using enzyme preparations

6.6. DISCUSSION

Cellulases find application in a wide variety of industries, but its use in second generation bioethanol production has brought it to the limelight. Though all the enzymes of cellulase system are required for hydrolysis, endoglucanase plays the major role. Studies conducted by Szijarto et al. (2011) showed that endoglucanase was the most efficient enzyme in causing liquefaction of lignocellulosic biomass. Recently, the demand for thermostable endoglucanase, capable of acting efficiently under harsh conditions of saccharification and other industrial processes is increasing. Usually, recombinant enzymes are preferred in industrial applications due to more consistency, less lot-to-lot variation, increased yield, specific activity and purity compared to native strain. Recombinant endoglucanase obtained in our work was stable over a wide range of temperature and pH and also showed significantly larger activity than the native strain (*Bacillus subtilis* MU S1). Thus, recombinant endoglucanase was used for saccharification studies.

Initially, five steam treated agro-wastes were examined for the hydrolysis rate. The highest sugar production was observed in JOR (33.4 %), followed by BP (23.1 %), PW (22 %) TH (21 %) and AH (14.9 %). The high content of lignin in other wastes may be the reason for low hydrolysis rate. As the enzyme used for hydrolysis is recombinant endoglucanase, it cannot degrade high lignin containing substrates, as it is deficient in accessory enzymes like ligninase, pectinase required for depolymerization of the complex structure and exposure of enzyme to cellulose. Bhandari et al. (2013) used cellulase

from *T. viride* for sugar production and found that jackfruit rind showed highest sugar production among the other fruit rinds (pineapple, watermelon and muskmelon) used by them. Begum and Alimon (2011) used sugarcane bagasse, sawdust and water hyacinth for cellulase induction and saccharification. They found that water hyacinth was the best substrate for cellulase production, but sugarcane baggase showed the highest saccharification.

Researchers have reported varying incubation times for optimum hydrolysis. The optimum reaction time for hydrolysis in all cases was 96 h and extending the reaction time had no much effect on hydrolysis. Similar incubation time for hydrolysis was observed by Begum and Alimon (2011) for saccharification of sugarcane bagasse, sawdust and water hyacinth. Bhandari et al. (2013) observed higher incubation time of 144 h for sugar production from jackfruit rind. Ali et al. (2015) observed maximum saccharification of pretreated bamboo biomass at 60 h. Singh et al. (2015) reported incubation time of 120 h for saccharification of *Parthenium hysterophorus* using endoglucanase from *Bacillus amyloliquefaciens SS35*. High saccharification in short incubation time is preferred.

Saccharification depends on various factors like pH, temperature, time of hydrolysis, enzyme and substrate concentration, enzyme stability and product inhibition. Hence, optimizing these critical factors is essential for improvement of enzymatic hydrolysis (Mahamud and Gomes 2011). Temperature and pH are vital factors that affect the enzymes catalytic ability. On optimization of these factors, saccharification of 34 % was obtained. The optimum

temperature and pH were found to be 50 °C and 5.0, which corresponds to the optimum condition of the cloned endoglucanase. The saccharification decreased with increase and decrease in pH and temperature beyond the optimum. Saccharification decreased greatly at temperature 60 °C (22.8%) and pH 4.0 (23.95 %). Same optimum conditions were observed by Sridevi et al. (2015), Ali et al. (2015) and Alrumman (2016) . Phadtare et al. (2017) reported saccharification of agro-wastes using a buffer of alkaline pH (pH 10.0) at 50 °C. Haq et al. (2015) found an optimum pH 7.0 and temperature 45 °C for saccharification of wheat straw and bagasse using recombinant endoglucanase.

The concentration of substrate used in saccharification reaction is an important factor. Concentration ranging from 5- 25 mg/ml was used for optimization. It was observed that the hydrolysis rate increased with increasing concentration of JOR and showed a maximum (37.8 %) at 15 mg/ml. Further increase decreased the saccharification percentage. The saccharification percentage reduced considerably to 7.15 % with 25 mg/ml JOR concentration. Increasing the substrate concentration resulted in an increase in cellulose available for hydrolysis, but as the concentration of the enzyme was not proportionally increased more cellulose was still left. This may have resulted in low glucose yield. The decrease in the aqueous movable space, end product inhibition or insufficient hydrolysis time may be other reasons for low glucose yield (Wen et al. 2004). A concentration of 2.5 % alkaline-treated sugarcane bagasse was reported as optimum by Mahamud and Gomes (2011), whereas Sridevi et al. (2015)

recorded 0.5 % alkali pretreated sawdust as optimum for saccharification.

Finally, the optimum concentration of the enzyme for saccharification of 15 mg/ml JOR was determined. Maximum saccharification of 41.1 % was observed when 18 U of recombinant endoglucanase was used. The hydrolysis decreased gradually with increase in enzyme concentration. A minimum saccharification of 18.85 % was observed on using 54 U of endoglucanase. As the cost of the enzyme is an important factor that limits the commercialization of second-generation bioethanol production, high saccharification using low concentrations of enzymes will be beneficial for industrial applications. The decrease in saccharification with an increase in the concentration of enzyme may be due to end product inhibition by glucose and cellobiose. Haq et al. (2015) observed maximum saccharification of wheat straw and bagasse at enzyme loading of 75 U. Whereas, Karmakar and Ray (2011b) obtained the highest hydrolysis on using 500 U of the enzyme and Phadtare et al. (2017), 20 U of recombinant endoglucanase for saccharification of different agro-wastes.

After optimization of process parameters, an overall 1.23 fold increase in saccharification percentage was observed. A maximum of 41.1 % saccharification of steam pretreated JOR was obtained at 96 h of hydrolysis, at temperature 50 ^{o}C and pH 5.0 with 15 mg/ml substrate, and 18 U of enzyme concentration. Compared to our study, lower saccharification was reported by Haq et al. (2015). They observed 5.12 % and 7.31 % saccharification of wheat straw and

bagasse respectively, at 45 °C, pH 7.0 using 75 U of cloned endoglucanase.

As the complete hydrolysis of lignocellulosic biomasses requires the participation of diverse hydrolytic enzymes, different enzyme preparations were tried for their saccharification efficiencies. It is frequently observed that, though β-glucosidase is essential for high saccharification, increased amount leads to increased glucose, which causes its feedback inhibition. This leads to decrease in overall saccharification percentage, due to the accumulation of a large amount of unhydrolyzed cellobiose and inhibition of endoglucanase by it (Holtzapple et al. 1990). Among the different enzyme preparations E3 (41 %) and CT1 (42.5 %) showed highest saccharification. The 1.5 % increase in saccharification by CT1 may be due to the presence of hydrolytic enzymes like xylanase, pectinase, ligninase besides cellulases (endoglucanase, exoglucanase and β-glucosidase) in E1. Whereas, the low saccharification in CT 2 (31.7 %) may be due to the high concentration of β-glucosidase in E2. Thus, recombinant endoglucanase (E3) was found to be effective independently and supplementation of accessory enzymes did not make much difference in its saccharification ability.

6.7. CONCLUSION

In the present study, the applicability of recombinant endoglucanase for saccharification of five locally available agro-wastes was determined. Among the wastes, the maximum saccharification was obtained for pretreated jackfruit outer rind. On

optimization studies, the saccharification percentage of JOR increased by 1.23 fold. The highest saccharification was obtained at temperature 50 °C, pH 5.0 with 15 mg/ml substrate and 18 U of recombinant endoglucanase. Trials with enzyme preparations containing recombinant endoglucanase did not show substantial improvement in saccharification as compared to recombinant endoglucanase alone. Thus, from the finding, we can conclude that the recombinant endoglucanase is highly efficient in saccharification of agro-waste and can find application in bioethanol production.

Cellulases are currently the third most important enzymes in the market, due to their wide range of applications. They find applications in industries like paper and pulp, laundry, textile, brewing, agriculture, food and feed. However, its application in biofuel industries has brought it into the limelight. Cellulases are multienzyme complexes consisting of endoglucanase, exoglucanase and β-glucosidase that act synergistically to bring about the complete degradation of cellulose to glucose. Among these endoglucanases are the prime enzymes required for initial liquefaction of cellulose. The main aim of the study was to isolate an efficient cellulolytic bacterium, improve its efficiency and find its applicability in saccharification of locally available agro-wastes.

The major finding of the study can be summarized as follows:

- Among the 32 cellulolytic bacterial strains isolated from different soil samples, four isolates showing the highest zone of clearance on CMC plates were selected and identified up to genus level using morphological and biochemical characters. All of these enzyme producers were obtained from protected forests of Munnar wildlife division.

- Two of the strains belonged to genus *Bacillus* (MU S1 and MU S3) and one each to *Staphylococcus* (MU S2) and *Corynebacterium* (MU S4). On assaying the endoglucanase activity of the four strains, highest activity (178.54 U/ml) was displayed by strain MU S1.

- The strain was identified up to species level by 16S rRNA gene sequencing. Based on BLAST analysis and phylogenetic tree results, the strain was found to belong to *Bacillus subtilis* and deposited in Genbank database with accession number KT715518.

- The optimum temperature and incubation condition for the strain *Bacillus subtilis* MU S1 was found to be 40 °C under agitation (140 rpm).

- The different cellulolytic enzymes produced by *Bacillus subtilis* MU S1 under the optimum growth conditions were determined. The maximum activity of endoglucanase (185.76 ± 4.6 U/ml), exoglucanase (151.64 ± 5.9 U/ml) and FPase (58.44 ± 3.1 U/ml) were seen at 24 h incubation, whereas β–glucosidase (156.02 ± 3.9 U/ml) showed the highest activity at 48 h incubation.

- The production of extracellular enzymes like amylase, pectinase, xylanase, protease, tannase and lignin-degrading enzymes by *B.subtilis* MU S1 was detected by plate assay.

- In order to improve the production of endoglucanase from *B.subtilis* MU S1, the physical parameters and media components were optimized using OFAT and RSM, respectively.

- The physical parameters (temperature and agitation speed) for maximum production of endoglucanase from *B.subtilis* MU S1 were found to be 40 °C and 150 rpm.

- Plackett-Burman design was used to screen the significant parameters that affect endoglucanase production from among ten selected independent variables. The concentration of unoptimized media components in g/l was; CMC (10.0), NaCl (6.0), $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (0.5), K_2HPO_4 (0.5), $MgSO_4$ (0.1), $CaCl_2$ (0.1), $NaNO_3$ (0.1) and Yeast extract (1.0) at pH 7.0.

- Among the six variables selected by PBD (CMC, yeast extract, NaCl, $MgSO_4$, $NaNO_3$ and pH), CMC, yeast extract, NaCl and pH showed a positive effect on endoglucanase production whereas $MgSO_4$ and $NaNO_3$ showed a negative effect. The concentrations of the significant factors with positive effect were optimized by central composite design. The optimal levels of the components were CMC 13.46 g/l, yeast extract 8.38 g/l and NaCl 6.31 g/l at pH 7.0.

- After OFAT and statistical optimization an overall 3.2 fold (566.62 U/ml) increase in activity was obtained compared to unoptimized condition (179.06 U/ml).

- As media influence endoglucanase production, three new media formulations were tried. Among these, media containing following components in g/l: CMC (10.0), NaCl (6.0), $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (0.5), Na_2HPO_4 (0.5), $MnSO_4$ (0.1),

$CaCO_3$ (0.1), KCl (0.1), Yeast extract (1.0) were found to produce higher activity than the unoptimized medium originally used.

- The application of few locally available agro-wastes (pepper waste (PW), banana peduncle (BP), arecanut husk (AH), tamarind husk (TH) and jackfruit outer rind (JOR)) as low cost alternative for synthetic carbon source was determined by replacing CMC in above medium with 1 % agro-waste.

- Among the agro-wastes, AH was found to induce highest endoglucanase production (328.8 U/ml) at 48 h of incubation. The strain produced all the hydrolytic enzymes like xylanase, β-glucosidase, FPase and avicelase using AH as sole carbon source.

- The AH medium was optimized by statistical methods, using a fixed amount of AH (1 %) as the sole carbon source. Using PBD, three factors (yeast extract, Na_2HPO_4, inoculum volume) were shown to have a positive influence on endoglucanase production. On CCD analysis the optimal concentrations of the components were yeast extract (8.38 g/l), Na_2HPO_4 (0.88 g/l) and inoculum volume (2.05 %).

- After statistical optimization of AH medium, an overall 2.2 fold (713.62 U/ml) increase in activity was obtained compared to unoptimized medium (328.8 U/ml).

- As for industrial applications, recombinant enzymes are desired, the endoglucanase gene (Egl) from *Bacillus subtilis* MU S1 was cloned in pET 22b vector and expressed in *E.coli* BL-21 (DE3).

- A nucleotide sequence of 1462 bp was obtained and on BLASTn analysis the sequence showed 99 % similarity to endoglucanase of *Bacillus subtilis* subsp. subtilis. The sequence was deposited in Genbank with accession number MG973067.

- The amino acid sequence, secondary and tertiary structures of endoglucanase were predicted using bioinformatics tools. The endoglucanase protein had 481 amino acids and a modular structure consisting of a catalytic (GH5) and non-catalytic domain (CBM3). The predicted molecular weight and theoretical pI were 53.22 kDa and 8.14 respectively. The secondary structure showed a high percentage of loops followed by strand and helix.

- On the expression of the protein, the highest activity was observed in intracellular fraction dissolved in supernatant followed by intracellular fraction dissolved in buffer and extracellular fraction (supernatant).

- The optimum IPTG concentration, temperature and incubation time for maximum production of recombinant endoglucanase was 1 mM, 25 ^{o}C and 16 h respectively. Under these optimized conditions, the endoglucanase activity (598.52 U/ ml) obtained

was 3.4 folds higher than the native strain (*Bacillus subtilis* MU S1).

- The recombinant endoglucanase was purified to homogeneity using Ni-NTA agarose affinity chromatography. The purification fold, yield and specific activity were 4.3, 45.7 % and 1624.51 U/mg respectively.
- The purified protein showed a single prominent band around 53-55 kDa on SDS PAGE. On zymogram analysis, a band of clearance was observed at a higher position (~100 kDa), which may be due to dimerization of endoglucanase protein in the native state.
- The recombinant endoglucanase was characterized. The optimum temperature and pH were found to be 50 ^{o}C and 5.0 respectively. The enzyme was stable over wide range of temperature (40 ^{o}C - 90 ^{o}C) and pH (5.0 -10.0).
- The endoglucanase activity was stimulated by metal ions like Mg^{2+}, $Mn^{2+}$and Fe^{2+}, slightly inhibited by Zn^{2+}, Ca^{2+}, $Na^{2+}$and strongly inhibited by Cu^{2+} and Hg^{2+}. Among all the additives, β-mercaptoethanol retained almost 100 % activity, whereas Triton X-100, SDS, EDTA and sodium hypochlorite inhibited the endoglucanase activity.
- For application in detergent industries, the compatibility of the enzyme with different commercial detergents was determined. Endoglucanase was found to be most stable in the presence of Tide, followed by Sunlight, Surf excel and Ariel.

- The enzyme could hydrolyze substrates other than CMC but at a lower rate. The activities towards other substrates were in the order filter paper > xylan > avicel > cellobiose.
- The K_m and V_{max}values of recombinant endoglucanase were found to be 3.97 mg/ml and 905.8 U/ml respectively.
- As second-generation bioethanol production is the topic of current interest, the application of recombinant endoglucanase in saccharification, which is the cost-limiting step in bioethanol production, was investigated using the same agro-wastes used earlier.
- Among the agro-wastes, highest saccharification was observed in JOR (33.4 %), followed by BP (23.1 %), PW (22 %), TH (21 %) and AH (14.9 %).
- The process parameters for increased saccharification of JOR were determined. A 1.23 fold increase in saccharification (maximum of 41.1 %) of steam pretreated JOR was obtained, at temperature 50 ^{o}C, pH 5.0 with 15 mg/ml substrate, 18 U of enzyme and at 96 h of hydrolysis.
- The efficiency of different enzyme cocktails on saccharification of pretreated JOR was tried. The highest saccharification (42.5 %) was obtained using a mixture of cloned endoglucanase and crude enzyme of *Bacillus subtilis* MU S1 in unoptimized medium (CT1). The saccharification was lower (31.7 %) in CT2 (cloned endoglucanase and crude enzyme of *Bacillus subtilis* MU S1 in optimized medium).

- The saccharification percentage obtained using recombinant endoglucanase (41 %) was almost on par with the enzyme cocktail.

Conclusions

The multifarious applications of the endoglucanase have prompted the researcher to look for efficient enzyme producers from unexplored environments. In this study we were successful in isolating efficient enzyme producers from protected forest soils, indicating that forest soil is certainly an affluent source for enzyme producers. Although, the endoglucanase production by *B.subtilis* MU S1 was improved by media optimization studies, for industrial applications recombinant enzymes are preferred. Thus the endoglucanase gene was cloned and over-expressed in *E.coli*. The properties of recombinant endoglucanase make it a potential candidate for applications in industries like detergent and biofuel. The study also demonstrated the efficient utilization of some of the locally available agro-wastes in Kerala including arecanut husk and jackfruit outerrind, for the cost-effective production of value-added products like enzymes and reducing sugars respectively. These wastes are usually dumped or burnt causing pollution problems. The bioconversion of these wastes increases the economic value of the wastes besides contributing to waste management. The study provides a lead for further research on the selected agro-wastes for biofuel production. Protein engineering and enzyme immobilization studies can further improve the catalytic efficiency, biochemical stability and also reusability of the enzyme.

SREENA C. P. “IMPROVEMENT OF BIOCATALYTIC EFFICACY OF ENDOGLUCANASE FROM INDIGENOUS STRAIN, BACILLUS SUBTILIS MU S1 AND ITS APPLICATION IN SACCHARIFICATION OF AGRO-WASTES”. THESIS. DEPARTMENT OF LIFE SCIENCES, UNIVERSITY OF CALICUT, 2018.

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