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Master's Thesis

Cultivation conditions for a moose (*Alces alces*) rumen bacterial isolate secreting cellulases

Master's Degree in Applied and Commercial Biotechnology

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ABBREVIATIONS

ABB	Anaerobic basal broth
LB	Luria Bertani media
CMC	Carboxy methyl cellulose
SDS- PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SI	Standard Inoculum
DNS	3, 5-dinitrosalicylic acid
CBM	Cellulase binding domain
CD	Catalytic domain
MEC	Multi enzyme complex
EGs	Endoglucanase
CBHs	Cellobiohydrolase
DP	Degree of polymerization
MRB_4	Moose rumen bacteria
SSF	Solid state fermentation
SmF	Submerged fermentation
MiMo	Mixed mode
MMC	Mixed mode chromatography
MiMo	Mixed Mode
CBB	Coomassie brilliant blue
AS	Ammonium sulfate

RCF	Relative centrifugal force
PJ	Potato juice
CASP	Clarified ammonium sulphate protein
MW	Molecular weight
RPM	Revolutions per minute
FI	Fermentation (first)
FII	Fermentation (second)
ANOVA	Analysis of variance
$CaCl_2$	Calcium chloride
pH	Potential of hydrogen

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Abstract

The lignocellulosic biomass is the major carbon source in the nature, but is not easily degraded because of its recalcitrant characteristic. The moose (*Alces alces*) primarily depends upon the lignocellulosic biomass as feed during winter season. Although, lignocellulosic biomass has recalcitrant properties, it is degraded and metabolized in the moose rumen and which microorganisms take part in this process are interesting and is drawing the attention. A cellulase secreting bacterial strain from moose rumen (MRB4) was used to evaluate its growth characteristic and cellulolytic properties. The cellulose is not degraded directly by microorganisms, but it is hydrolysed by the cellulolytic enzymes (cellulases) secreted by the microorganisms. The enzymes are inducible by varieties of substances then secreted by microorganisms. The cellulase is an enzyme complex (endoglucanase, exoglucanase and beta-glucanase) that act synergistically to degrade cellulose into its simple form, glucose. The main goal of this study was to explore conditions for optimal growth and maximum enzyme (CMCase) secretion with MRB4 using various supplements and response factors. The MRB4 isolates were cultured in both ABB media and LB media supplemented with Tween-20 and either potato rasp or cellobiose as inducer. Carboxy methyl cellulose (CMC), $CaCl_2$, and temperature were tested as response factors in a fractional factorial Design of Experiments. The effects of inducer and factors for optimal cell density and the CMCase activity were analysed by reducing sugar assay and in zymography using CMC as substrate. Based on the results obtained from the fermentation I and DoE design, the fermentation II was carried on the basis of DoE for further confirmation. The possibility of capturing the enzyme from the culture broth was also tested using mixed mode chromatography resin.

The cultivation performed in small scale and bench scale with CMC, cellobisoe, rasp revealed that CMC only supported for increased cell density, but not CMCase activity, which decreased after prolonged culture (24 hours). The cellobiose (0.5 %) had a positive effect on CMCasea activity with maximum level after 9 to 12 hours of incubation, but not in cell density. Contour plots from DoE analysis predicting optimal cell density and CMCase activity of MRB_4 showed that $CaCl_2$ and CMC with concentration around 15 mg ml^{-1} and 1.5 % w/v respectively, can enhance the cell density, while the highest CMCase activity can be obtained during higher $CaCl_2$ concentration (25 mg ml^{-1}), but lower CMC concentration (0.25 % w/v). The effects plots showed that $CaCl_2$, CMC and temperature all had significant and positive effects on bacterial growth although; the level of CMCase activity was very low.

The ammonium sulfate pellet obtained by treating the culture supernatant was further purified by cationic mixed mode chromatography applying different conductivity (5 - 34.1 mS cm^{-1}) and loading volume (5 - 40 ml). Highest protein yield was observed for medium salt (10 mS cm^{-1}). The captured enzyme after elution in the elution fraction showed the CMCase activity and active halo bands in SDS-PAGE zymography. The SDS-PAGE zymography analysis demonstrated the active halo bands at approximately 50 kDa to 60 kDa. A 20 kDa-25 kDa band, however only appeared in 17X (10 mS cm-1) diluted MiMo resin fractions.

It is concluded that MRB4 is a cellulolytic bacteria and it secretes inducible cellulase (endoglucanase) stimulated by variety of supplements and inducers in defined concentration and conditions. The MRB4 isolates can be the valuable source of lignocellulose degrading enzymes.

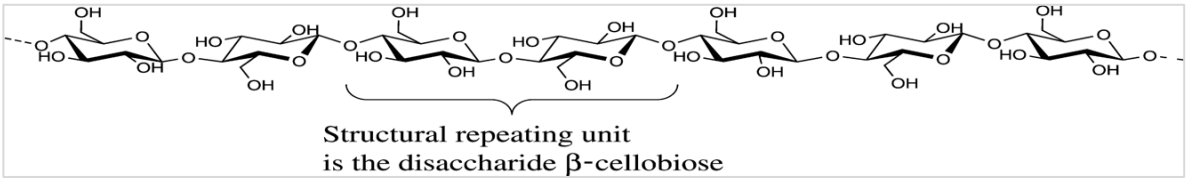
1. INTRODUCTION

1.1 Plant (lignocellulosic) biomass

Lignocellulosic biomass is the most abundant biopolymer on the earth (V. Juturu & Wu, 2012) which is made up of organic matters such as cellulose, hemicellulose, lignin, pectin. The organic matters carry around 95 % of total plant mass while inorganic matters such as calcium, sodium, silicon, phosphorous, magnesium and extractives carry rest of the plant biomass in dry weight (Dai, Saayman, Grace, & Ellis, 2015).

The cellulose is long chain linear homo-polymers of beta D-glucose which is connected by beta-1,4-glyosidic linkage (Lakhundi, Siddiqui, & Khan, 2015). The beta-1,4 linked glucose is the chemical repeating units, while structural repeating units are beta-cellobiose in cellulose (Varrot et al., 2003).

A



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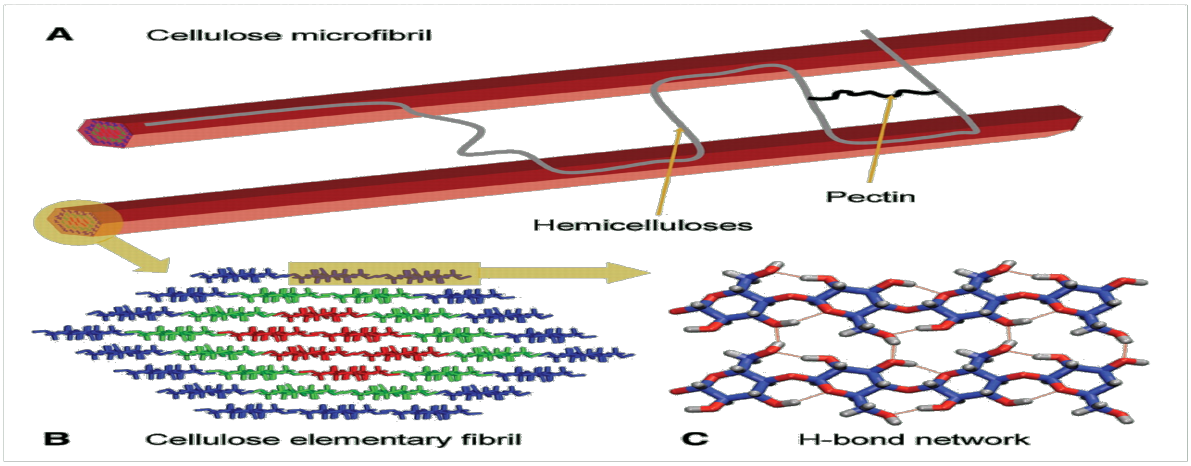


Figure 1: Chemical and structural structure of cellulose. (A) Chemical structure of cellulose formed by linear beta-1,4 linked glucose monomers and cellobiose is made up of two glucose monomers (Varrot et al., 2003). (B) Schematic representation, orientation and organization of major polysaccharides in the plant cell wall where A, B and C are cellulose micro fibrils, cellulose elementary fibril and H-bond network between the elementary fibrils to create high tensile strength respectively. Sources; (Himmel et al., 2007).

The glucose monomers in cellulose are tightly bounded and hold by hydrogen bounding and van der Waals forces in crystalline structure called elementary micro fibril which makes it insoluble to most of the solvents and increases resistivity to microbial degradation (Lakhundi et al., 2015; Varrot et al., 2003). Aggregates of cellulose elementary fibril is called microfibril which is depicted in the figure 1. Cellulose also has hydrophobic surface that acts as the protective layers and prevents diffusion of cellulolytic enzymes thus, protect the entire cellulose (Jørgensen, Kristensen, & Felby, 2007).

Cellulose is chemically homogeneous (figure 1 A), but structurally diverse or heterogeneous as it exists in both crystalline as well as amorphous form. It is differed with the starch primarily because of its crystallization properties and it has a linear polymer chain. Cellulose in crystalline form is less susceptible to degradation and higher in proportion, but the amorphous part is more susceptible to the enzymatic degradation as well as present in low proportion (Kumar, Barrett, Delwiche, & Stroeve, 2009).

The synthesis of cellulose by polymerization of glucose monomers in the plant cell is derived from photosynthetic process which is accomplished in presence of solar energy, cellulose synthase enzyme and UDP at the plasma membrane (Dai et al., 2015). Though cellulose is the major component of lignocellulosic biomass, it is also produced by other environmental components such as green algae (*Valonia and Micrasteriase*), slime mold *Dictyostelium,* bacteria *(Acetobacter xylinum),* marine animals *(Halocynthia)* and other animals *(Tunicates) etc. (Lakhundi et al., 2015).* The percentage of cellulose in plant biomass depends mainly on the types of wood, where it is growing, age and environmental conditions, but generally hardwood stems and softwood stems contain 40 - 55 % cellulose while cotton seed hairs and paper contains 80 - 95 % cellulose (Kumar et al., 2009).

Unlike cellulose, hemicellulose are complex hetero polymers and consists of short, random, amorphous, and highly branch chain of monomeric residues with lower degree of polymerization (~100 - 200). Hemicellulose is more susceptible to chemical and the enzymatic degradation because of its amorphous structures (Zabed, Sahu, Boyce, & Faruq, 2016). The major monosaccharides in hemicellulose are pentose (D- Xylose, L- arabinose and rhamnose), hexose (D-glucose, D-galactose and D-mannose) and other uronic acids for example: 4-0-methylglucoronic, D glucuronic and D-galacturonic acids (Jørgensen et al., 2007). Lignin is an unstructured, complexed and cross-linked aromatic polymers of phenol propane units in the cell wall of the biomass. The three phenolic monomers in lignin are phenyl propionic

alcohol, coumaroyl, conferyl and sinapyl alcohol. Lignin embeds cellulose and hemicellulose thereby providing structural support as well as protection against microbial and chemical attack (Kumar et al., 2009).

1.2 Moose rumen bacteria

The moose (*Alces alces*) is the herbivorous ruminant and their main dietary sources are varieties of hardwoods and deciduous species (e.g. willow, aspen, ash, maple) during the winter seasons, but in summer seasons aquatic vegetation and swamps are main dilatory sources (Ishaq & Wright, 2012). Like all ruminant, the moose digestive tract is divided into four compartments as rumen, reticulum, omasum and abomasum. Out of these compartments, rumen and reticulum are rich in complex consortia of microorganisms and are responsible for the breakdown of plant fibers, primarily cellulose because these animals cannot degrade on its own (Ishaq & Wright, 2012; van Dyk, Sakka, Sakka, & Pletschke, 2009). The moose rumen is full of several sorts of feeds content with varieties of anaerobic microorganisms (bacteria, Protista and fungi) which acts as bioreactor and breakdown the feeds content into the volatile fatty acids that are the major energy source for all the ruminant (Henderson et al., 2015). Moose rumen contains verities of microbes and these microbes carry different metabolic properties such as cellulolytic, amylolytic and proteolytic and few of them are involved in the metabolism of the metabolic products of other microbes (Henderson et al., 2015). However, the moose gut microbiota varies with diet, age and habitat, but the major microbes found in the moose rumen are; *Prevotella, Butyrivibrio, Ruminococcus, Bacteriodales* and *Clostridiales* (Svartström et al., 2017).

In previous study, a moose rumen complex consortium of microorganisms was known as microbiota and that were categorized by using the simple microbiological and traditional culturing techniques. The moose rumen sample was taken from the moose of Alaska, Norway and the bacterial colonies were categorized after analysis into the *Streptococcus bovis* (21 strains), *Butyrivibrio fibrisolvens* (9 strains), *Lachnospira multiparus* (7 strains), and *Selenomonas ruminantium* (2 strains) (Dehority, 1986). The second study was done using second generation PhyloChip microarray to identify and categorize the types of bacteria present in the moose rumen. The second-generation technique uses the 16S rRNA sequencing techniques to distinguish the bacteria from a mix rumen microbial sample (Ishaq & Wright, 2012).

1.3 Moose rumen *bacillus* as cellulase producing bacteria

However, Protista occupy 50 % biomass in the moose rumen, but it is generally dominated by the bacterial cells in term of number (Svartström et al., 2017). The variation in the bacterial rumen composition is common in animals (moose) from different regions, most likely because of different habitat, diet and climate (Henderson et al., 2015). Among other moose rumen bacteria, the *Bacillus* are major gram positive, rod shaped, non-pathogenic (except: *Bacillus anthraces and Bacillus cereus*), facultative anaerobic microorganisms used for the production of different sorts of enzymes, proteins, antibiotics, insecticides and other biochemical in industrial level (van Dyk et al., 2009). Most of the *Bacillus species* produce endospores which are strictly resistant to the adverse environmental condition. Predominantly, the aerobic strains of *Bacillus* are motile by peritrichous flagella (van Dyk et al., 2009). *Bacillus licheniformis* are recognized as the higher amount of extracellular protein producer together with higher growth rate in short fermentation cycle. So its possibility and utilization in industrial level is increased day by day and the complete genome analysis of several types of *Bacillus* have been sequenced to understand the physiology, biochemistry and genetic variation as well as similarity (Archana Sharma & Satyanarayana, 2013).

The recent taxonomic study reveals that *B. licheniformis , B. subtilis and B. amyloliquefaciens* are closely related on the basis of results obtained by performing 16S rDNA and 16S-23S intergenic spacer nucleotide sequence (ITS) (Rey et al., 2004). The genomic analysis of the *B. licheniformis* find out that it has 4, 222.748 long base pair chromosome which contains 4, 286 genes. The chromosome is made up of 46.2 % G+C bases, seven rRNA operons, 72 tRNA genes and 87.9 % part of the gene is occupied by the protein coding regions (Veith et al., 2004). *B. licheniformis* secrets both extracellular endoglucanases and xylanase during its growth in the media like ABB and BHI, etc. (van Dyk, Sakka, Sakka, & Pletschke, 2010b). The secreted proteins can be predicted and detected on the basis of genomics and the chemical and biochemical test, while its type and secretion mechanism can be deduced by the proteomics (Voigt et al., 2009). Similar to *B. subtilis*, the *B. licheniformis* includes four sorts of protein secretary pathway; the secretary protein translocation (Sec) pathway, twin-arginine translocation (Tat) pathway, pseudopilin pathway and ABC transporters pathway. The Sec. pathway associates with unfolded proteins transportation, while strongly folded proteins are transported through the tat pathways to the cytoplasm (Van Dijl et al., 2002). The tat, pseudopilin and ABC transporter pathways are recognized as the specialized and protein

specific pathways. When early protein secreted in the nucleus before exported into the extracellular medium, the N-terminal signal peptides help to direct and detect the right translocation pathway. Eventually, the signal peptides are detached from the protein during the translocation from cytoplasm to the extracellular medium. The exported proteins generally occur in two forms; either in free form or in the combined form with cytoplasmic membrane proteins. e.g. lipoproteins (Van Dijl et al., 2002). Even though *B. licheniformis* and *B. subtilis* genome have organizational and functional similarities and also most of the genes are orthologues to each other, but there is variation in gene orientation such as; location of prophages, transposable elements, number of extracellular enzymes, and secondary metabolic pathway (Archana Sharma & Satyanarayana, 2013). (Archana Sharma & Satyanarayana, 2013) studied on the *bacillus* genome especially *B. licheniformis* and they illustrate few genes, designated as pgIH, bgIC, yjeA. They recapitulate that these genes are essential and involved in extracellular protein production. In *B. licheniformis*, the intra and extracellular proteins secretion is guided by regulatory genes and specialized secretary system such as regulatory DNA motives, glycosylate bypass, anaerobic ribonucleotide reductase which makes it possible to grow on acetate, and glucose anaerobically (Archana Sharma & Satyanarayana, 2013).

1.4 Cellulolytic enzymes (cellulases)

Cellulose is the most abundant and naturally fixed form of carbon source which is primarily produced in the plants cell wall. Even though it is ubiquitous in nature, very limited numbers of organisms able to degrade it, which is because of its recalcitrant nature (D. B. Wilson, 2008). Generally, the common and primary cellulolytic microorganisms are fungi and bacteria, but it is also produced by the other organisms such as; nematodes, protozoa, insects, and mollusks. In case of ruminants (moose), the ruminal microorganism such as; different bacteria, fungi or protozoa degrade cellulose to energy generation (Wilson, 2011). The cellulose is impermeable and complex to the bacterial cells, thus it cannot be utilized directly as energy source in the living system. So, most of the cellulolytic bacteria and fungi secretes extracellular cellulase enzyme to breakdown cellulose into simple sugars (D-glucose) which are soluble and easily transported into the cell then metabolized (Wilson, 2011). Cellulase enzyme degrades the cellulose by hydrolyzing beta-1,4-glyosidic linkages between the glucose monomers. The glucose monomers of cellulose are linked together by beta-1,4 glyosidic bond as well as with hydrogen bonds and ionic strength that make it highly recalcitrant to microbial degradation (Bayer, Chanzy, Lamed, & Shoham, 1998). The enzymatic hydrolysis of cellulose

is possible, and it occurs in multi-enzyme complex fashion, where they act synergistically to hydrolyze beta-1,4-glyosidic bonds between glucose monomer of amorphous cellulose. The enzymes included in this system are endo-ß-1,4-glucanase, cellobiohydrolase and ß-glucosidase (Xie et al., 2007). Cellulase is induced in presence of certain stimulating substances in a large number of microorganisms such as; bacteria and verities of fungus as well as other microorganisms. These microorganisms secrets cellulase during their growth to degrade the cellulosic substrate into its simple form (Kuhad, Gupta, & Singh, 2011).

1.4.1 Organization of cellulases

Cellulases are modular enzymes consists of domains or modules which are folded differently and consider as the structurally and functionally discrete units of the enzymes (Bayer, Chanzy, et al., 1998). Similar to the other enzymes which comprise of Catalytic domains (CDs) and Substrate **b**inding **d**omains (SBDs) with flexible linker, rich in serine and threonine amino acids. Cellulases are also made up of these fundamental enzyme domains, but the domains are designated differently in it. For example, the SBDs in cellulose is designated as Cellulose **b**inding **d**omains (CBDs) as it was firstly discovered in cellulase enzymes. Later on, it is designated as Carbohydrate binding modules (CBM) which is responsible to direct the enzyme towards the cellulose so that the CDs can act actively on cellulose molecules (Wilson, 2011). Most of aerobic microorganisms use free extracellular cellulases that acts synergistically to degrade cellulose, but many anaerobic microorganisms degrade the cellulose via complexed cellulase system known as cellulosome (Wilson, 2011).

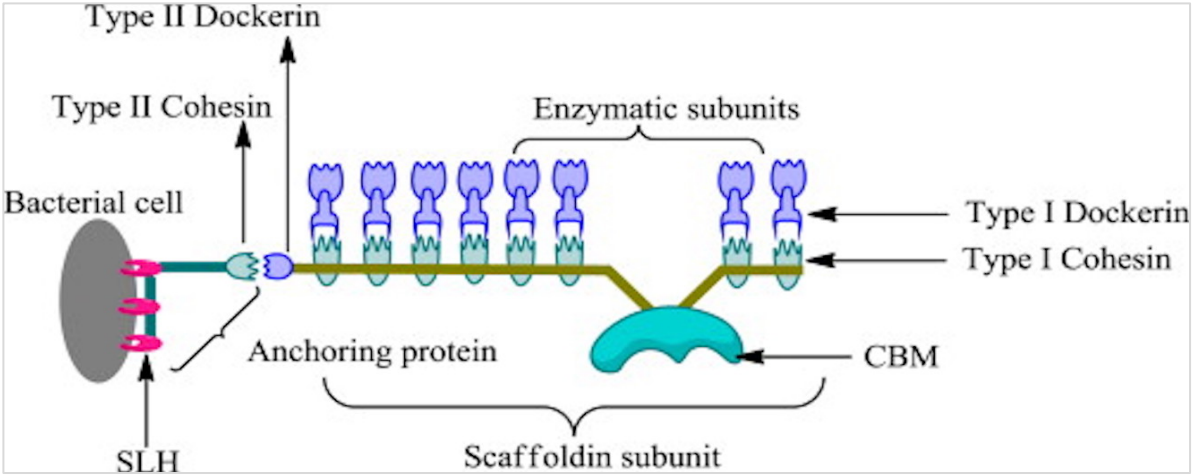


Figure 2: The simplified module of bacterial cellulosome architecture with different subunits namely scaffoldin, dockerin and cohesion and enzymatic subunit. Source., (Veeresh Juturu & Wu, 2014).

Even though the end product of cellulose degradation from both aerobic and anaerobic fungal and bacterial cellulase system is glucose monomers, but the anaerobic bacterial cellulase system is structurally more complex than the aerobic fungal and bacterial cellulase (free cellulase) system due to cellulosome (Kuhad et al., 2011). The fungal cellulase made up of Catalytic domain and Cellulose binding domain which are joined by polylinker region to the N-terminal of the CDs. The cellulosome and the free cellulase system are differ in structure and mechanism of action with each other due to variation in the structural components of cellulosomes- cohesin containing scaffolding and dockerin containing enzymes. The free cellulases system rich in CBMs lacking dockerin domain, while cellulosome contains dockerin domain direct to the cellulose degradation without CBMs (Kuhad et al., 2011).

The cellulosome is firstly discovered from the anaerobic thermophilic cellulolytic bacteria, *Clostridium thermocellum* which then known as the multi-enzyme complex and recognized as alternative mechanism of cellulose breakdown (Bayer, Shimon, Shoham, & Lamed, 1998). The cellulosome system is organized by non-catalytic scaffoldin subunits that comprise of carbohydrate binding model and the multi-modular cohesion-dockerin complex interaction. At microscopic level, the cellulosome consists of enzymatic subunits and non-enzymatic scaffoldin proteins with cellulose binding domains or carbohydrate binding module that binds strongly to the cellulose substrate and facilitates cellulose degradation (Yoav et al., 2017). Cohesin and dockerin are important units of scaffoldin proteins, where one side of dockerin binds with enzymatic units and another side to the cohesion unit, connecting protein via the anchoring protein known as S-layer homology module (SLH) that facilitate the connection to the bacterial cell surface (Bayer, Belaich, Shoham, & Lamed, 2004). According to (Bayer et al., 2004), the bacterial cellulosome system not only categorized into two types on the basis of presence of multiple sorts of scaffoldins (e.g. *C. thermocellum*) and single scaffoldin (e.g. *C. cellulovorans*), but also characterized on the basis of primary scaffoldin consists of CBM and enzymatic subunits with an anchoring scaffoldin connecting to bacterial cell surface by SLH models. On the basis of variation in composition of enzymatic subunits and scafoldin subunits, cellulosome system is considered as heterogeneous in nature and it differs among the bacterial species (Veeresh Juturu & Wu, 2014).

1.4.2 Classification of cellulases

The microbial cellulases are either cell associated or extracellular and are differed due to their mode of action towards the cellulose (Amita Sharma, Tewari, Rana, Soni, & Soni, 2016).

(Sadhu & Maiti, 2013) classified cellulase system based on biochemical analysis and their mode of catalytic action into the following categories.

- Endoglucanases or Endo-1, 4-ß-D-Glucan Glucanohydrolases (EC 3.2.1.4)
- It is active against the amorphous site of the cellulose forming oligosaccharides that are variable in length and degrades the soluble derivatives of cellulose such as CMC and cellooligosaccharides.
- Exoglucanase or 1,4-ß-D-Glucan Cellobiohydrolases (Cellobiohydrolases) (E C 3.2.1.91)

  It acts on the reducing as well as non-reducing ends of the cellulose and it generates glucose and cellobiose. These enzymes are active against the crystalline cellulose, amorphous cellulose and cellooligosaccharides.
- Exogluconase or 1,4-ß-D Oligoglucan Cellobiohydrolases (cellodextrinases) (E C 3.2.2.74)

  It acts on the cllooligosaccharides and liberates cellobiose, but inactive against amorphous cellulose or CMC. Both acts on reducing and non-reducing ends.
- ß – Glucosidases or ß-D-Glucoside Glucohydrolases (E C 3.2.1.21)

  Glucose from non-reducing ends of cellobiose and cellodextrins is generated by ß-Glucosidases is the final product of the cellulose degradation and is metabolized by the cell. It does not show any response to the amorphous and crystalline cellulose.
- Cellobiose Posphorylase or Cellobiase (E C 2.4.1.49)

  It is also known as Orthophosphate Alfa-D-Glucosyl Transferase. It involves in the reversible phosphorylitic cleavage of cellobiose into glucose units. Initially, it was discovered in the *Ruminococcus flavefacience* cells (Ayers, 1959).
- Cellodextrin phosphorylase (E C 2.4.1.49)

  It was firstly reported in the cells of *Clostridium thermocellulam (*Sheth & Alexander, 1969) and is also known as 1,4-ß-D-Oligoglucan Orthophosphate Alfa-D-Glucosyl Transferase which catalyze the reversible phosphorylitic cleavage of cellodextrins to glucose. It is inactive towards cellobisoe.
- Cellobiose Epimerase (E C 5.1.3.11)

  It was found in the cells of *Ruminococcus albus* (Tyler & Leatherwood, 1967) epimerizes the disaccharides like cellobiose into 4-0-ß-D-glucosylmannose.

1.4.3 Mechanism of cellulases

The cellulolytic bacteria or fungi secretes cellulase to degrade cellulose molecules into its simpler form because of its insoluble, impermeable nature and structural complexity that hinder it to metabolize directly as energy sources in the cells. In order to degrade cellulose into its simpler form, the specific enzyme known as cellulase acts on it and convert it into glucose molecules which is easily metabolized and soluble in nature (Wilson, 2008). Cellulase shows normal activity towards the low molecular weight soluble cellulose substrates, but it shows variable activity towards the insoluble cellulose substrate because of its heterogeneity (Wilson, 2008). The common enzymes that involve in synergistic action of cellulose degradation are endoglucanase, exoglucanase/ cellobiohydrolase (CBHs) and ß-glucosidase (Xie et al., 2007).

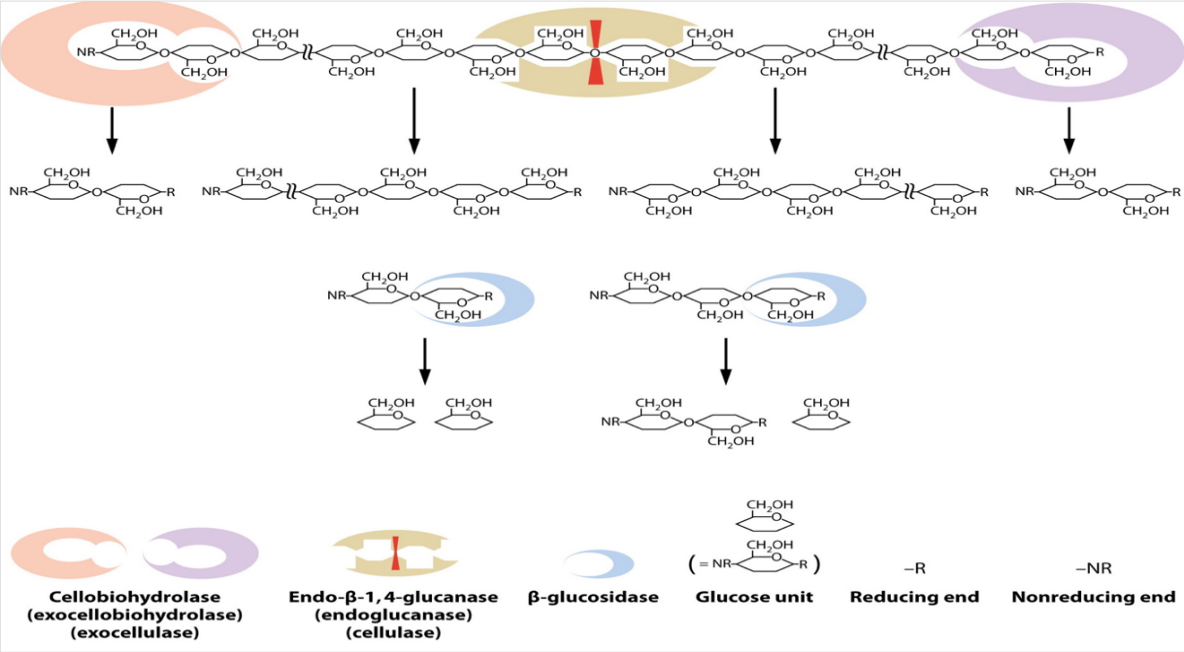


Figure 3: Cellulolytic enzymes involve in the breakdown of the cellulose into glucose. Endoglucanase (denoted as orange and purple color) randomly attacks the internal glyosidic (beta-1,4-glyosidic) bonds of cellulose and resulting into smaller cellulose fragments which are then breakdown into the cellobiose by exoglucanase (denoted as light-yellow), acting on both reducing and non-reducing ends of the cellulose fragments. Eventually, ß-glucosidase (denoted as blue color) hydrolyzes cellobiose into the glucose residues. Source, (Watanabe & Tokuda, 2010).

The details overview of the cellulolytic enzyme synergistic activity is represented in the Figure 4 (Watanabe & Tokuda, 2010). The most well-known synergism of the cellulolytic enzymes is end-exo synergy and exo-exo synergy. In endo-exo synergy, endogluconase cuts cellulose randomly producing smaller cellulose fragments with reducing and non-reducing ends and

then, these ends attack by exonuclease or CBHs progressively to generate cellobiose. Finally, ß-glucosidase attacks cellobiose to produce glucose monomers (Dashtban, Schraft, & Qin, 2009). While exo-exo synergy is occurred between two CBHs where one CBHs act in one reducing ends and another complementary CBHs acts on another non-reducing ends to generates glucose monomers (Jalak, Kurasin, Teugjas, & Valjamae, 2012). Since cellulose is heterogeneous in nature that results in variation in the rate of enzymatic hydrolysis (Lynd et al., 2008). The rate of cellulose breakdown is also affected during the course of cellulose hydrolysis since the amorphous region of the cellulsoe is degraded initially at faster rate than the crystalline region, which are hydrolyzed slowly. (Lynd, Weimer, Van Zyl, & Pretorius, 2002).

However, cellulolytic hydrolysis accomplished either in complexed or in non-complexed manner with or without synergism respectively, but both of them are inhibited by several sorts of inhibiters during the course of cellulolytic hydrolysis (Andrić, Meyer, Jensen, & Dam-Johansen, 2010).

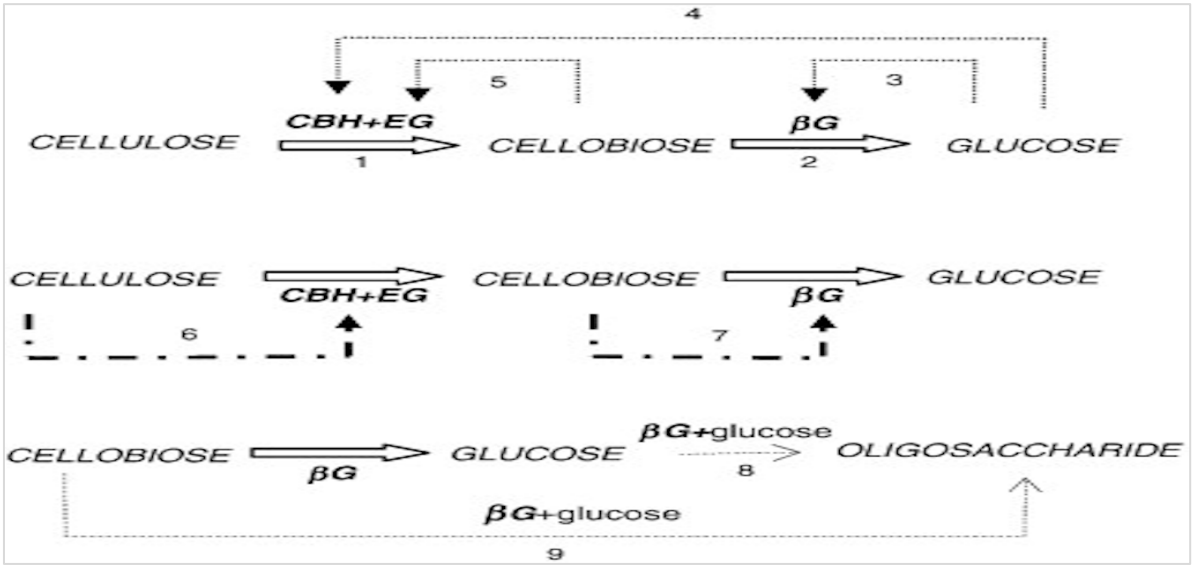


Figure 4: The Schematic representation of the cellulolytic hydrolysis and the types of inhibition including major kinetic pathways with synergism. The types of reactions are denoted as; (A) synergistic reaction 1, 2, (B) product and glucose inhibition 3 and 4, cellobiose inhibition 5, (C) substrate inhibition 6, 7, and (E) transglycosylation 8, 9. Source, (Andrić et al., 2010)

Generally, the type of inhibition is categorized based on the name of inhibiters involve and effect of inhibitors in the chain of reactions. For instance, if substrate and product inhibit the reaction then the inhibition is known as substrate and product inhibition respectively. Furthermore, it was categorized as competitive, non-competitive, uncompetitive, mixed, partial, allosteric etc. based on the activity of inhibiters (Andrić et al., 2010). In case of

cellulolytic reaction, the cellulase enzyme is inhibited by the partial inhibition known as parabolic inhibition (Bezerra & Dias, 2004) where the glucose and the cellobiose are key inhibiters which inhibit the entire chain of reactions (Bayer et al., 2004).

1.5 Cellulase production via fermentation

Now a days, the cellulase production becoming wide and hot research topic globally so several investigators and researchers are concerning towards the cellulase enzymes and its production using different sorts of substrate like lignocellulosic biomass (Sadhu & Maiti, 2013). But the key and challenging factor for cellulase production is low enzyme yield or titer that's why several research workers focusing for the higher yield of cellulase using better bioprocess technologies, selecting cheap and crude raw materials as substrates and including higher yield, fast growing and safe microorganisms (Singhania, Sukumaran, Patel, Larroche, & Pandey, 2010). The majority of reports on microbial fermentation for production of cellulase includes two techniques such as solid-state fermentation and submerged fermentation. Fermentation is the conversion of complex biological substrate which are rich in carbon source by using suitable microorganisms into simple compounds (Sadhu & Maiti, 2013). During metabolic breakdown of the complex substrate, the primary metabolites (usual product) together with secondary metabolites are produced. The secondary metabolites currently known as the bioactive compounds including several sorts of gases (Co_2), alcohol, peptides, enzymes, growth factors, antibiotics and other different compounds (Subramaniyam & Vimala, 2012). Two broad processes namely Solid-state fermentation (SSF) and Submerged fermentation (SmF) are the crucial technique that has immense important in bioprocess technology (Sadhu & Maiti, 2013).

1.5.1 Solid-state fermentation (SSF)

SSF is the fermentation techniques which is carried out in the absence or nearly absence conditions of water with solid substrate like agriculture wests, barn bagasse, paper pulp and other lignocellulosic waste. SSF is established and becoming interesting for cellulase production because of its cost effective and highly concentrated cellulase yield using the fungal microorganisms (Tengerdy, 1996). Filamentous fungi such as *T. reesei, A. niger*, *Penicillum* sp. etc. are the most referent and frequently used species for the production of cellulase using SSF (Mekala, Singhania, Sukumaran, & Pandey, 2008). (Mekala et al., 2008),

find out that *T. reesei* has better cellulase yield in SSF cultures in comparison to liquid cultivation. When compared the cellulase production from SSF and SmF system the conclusion will be that the cellulase production cost can be reduced to 10-folds down by using SmF technique. They also concluded that the 10 folds reduction in cost is due to higher cellulase production concentration that decreases the steps in downstream processing (Tengerdy, 1996). Even though this sorts of fermentation techniques take a long time to ferment the substrates, but the bioactive compounds can be released in controlled manner (Babu & Satyanarayana, 1996). Fungi are suitable for this type of fermentation technique because it requires less water/moister content (Subramaniyam & Vimala, 2012).

1.5.2 Submerged fermentation (SmF)

Submerged fermentation uses liquids and semi-liquids as the substrates e.g. molasses and broths. In this technique, the rate of substrate utilization and the release of bioactive compounds in fermentation broth is fast and easy, though the production yield very low. This technique is suitable for the fermentation of substrate using bacterial organisms that requires higher amount of water and the purification techniques is easier as compared to the SSF (Subramaniyam & Vimala, 2012). Cellulase production in cultures is highly influenced by various parameters such as types of cellulosic substrate, temperature, pH of media, presence of nutrient, and types of supplemented inducer etc. So, for better understanding about all these factors that influence the bacterial growth and the total effects of the inducer for optimal bacterial growth and maximum cellulase production is essential for the higher yield of product (Singhania et al., 2010). The induction and the repression processes are interrelated with microbial cellulase production. For illustrations, *T. reesei* and *Bacillus sp.* produce inducible cellulase and the higher yield is obtained in presence of cellulose as main carbon sources (Singhania et al., 2010).

1.6 Aim of study

The research work was based on the project of INN, Department of Biotechnology, Campus Hamar that aims to find out optimal bacterial growth together with maximum enzyme secretion of MRB4 isolates using verities of cellulosic biomass efficiently and cost effectively that eventually lead to establish commercially viable cellulase enzymes production system.

The aim of this study was to investigate technique for cultivation and secretion of the endogluconase using varieties of commercial media as well as industrial wests with or without supplementation of effective inducer that changes the track and suggest optimal bacterial growth with maximum cellulase secretion. Furthermore, the goal of secreting cellulose degrading enzymes using bacterial isolate was to establish an improved hydrolysis of cellulosic biomass to fermentable sugars. The cellulolytic potential of MRB4 extracellular cellulase known as CMCase was detected and analyzed by using CMC as a substrate. The bacterial strains were cultivated on several lignocellulosic biomasses which act as carbon sources supplements such as, CMC, rasp and cellobiose, etc. to generate cellulose degrading endoglucanase.

The secreted enzymes supernatant was tested and analyzed in different hydrolysis experiments such as DNS assay, SDS-PAGE zymography and chromatography to conform its presence, find out its types, detect its activity and sensitivity to the substrates for enzyme secretion.

Objective of the study were:

- To find out the better growth characteristics and higher enzyme secretion of MRB4 with various of supplements during its cultivation.
- Set up of Design of Experiments for optimal bacterial growth and maximum enzyme secretion using RSM.
- Identification analysis and evaluation of MRB4 inducible enzyme (CMCase) using biochemical, molecular as well as routine techniques.
- Implementation of purification technique (Mixed mode chromatography) for separation and capture of CMCase from processed culture supernatant.

2. MATERIALS AND METHODS

2.1 Inoculum preparation

A bacterial isolate from moose rumen designated as MRB4 (Moose Rumen Bacteria no. 4) was stored at -80 °C in the laboratory. The bacteria were isolated at University of Bergen (prof. V. Bakken, pers. comm), and initially characterized as similar to *Bacillus licheniformis* (Sanusi, 2013) and (Rabiul, 2016) A lyophilized sample of MRB4 was transferred to Anaerobic Basal Broth (ABB) media and cultivated under stirring at 30 °C. The overnight culture was than aliquoted 1ml in sterile micro-centrifuge tubes and stored at -80 °C. Tubes of the standard inoculum were retrieved as required for raising working cultures while performing experiments in the laboratory.

2.2 Chemicals and biochemicals

Anaerobic basal broth (CM0957, Oxoid), D (+) cellobiose (219458-25GM), sodium carboxy methyl cellulose (C4888), D (+) glucose (G-7528), birchwood xylan (X4252), ammonium persulfate (0486-25G), Congo red (C6767-25G), polyethylene glycol (PEG), (58H0071) were product of SIGMA, (St. Louis, MO, USA). The other chemicals - sodium citrate (A0534248441), sodium chloride (K48737404722), Coomassie brilliant blue R-250(0472-10G), glycerol 85 % (Z0313094406), sodium chloride (K48737404722), Sodium Acetate (AM0853565335) were obtained from standard lab suppliers (VWR, Norway).

Cellulase from *Trichoderma reesei* ATCC26921(SIGMA) (C2730), and cellulase from Aspergillus species (SIGMA) (C2605) were the product of Sigma, whereas cellulase NS-81210 (CZP0011) and Celluclast (1500NCU/G), 2880 were the product of Novozymes, Bagsvaerd Denmark. DSM Metha PLUS L100, a hemicellulose enzyme complex was product of DSM (Herleen, the Netherlands). All mentioned enzymes were used as the reference enzymes in the electrophoresis. Colored pre-stained protein standard/Protein ladder; (Protein ladder, Precision Plus Protein™ colour standard, 1ml, broad range (11-245 kDa) was used for size standardization of the cellulase bands in SDS-PAGE.

The dried and autoclaved rasp and Potato juice (PL) was used as carbon source and liquid media for the growth of MRB4 respectively which was obtained from the industry (Brumandal, Norway).

2.3 Chromatography column

A 5ml of cationic mixed mode (MMC) resin (Capto™ MMC HiTrap™, GE Life Science, Uppsala, Sweden) was used in the experimental work. The column resin was made with a ligand that covers multimodal functionality (Figure 5). Targeted protein will bind to the ligand with multiple interaction. The potential interactions within the column to capture protein molecules are ionic interaction, hydrophobic and hydrophilic bonding.

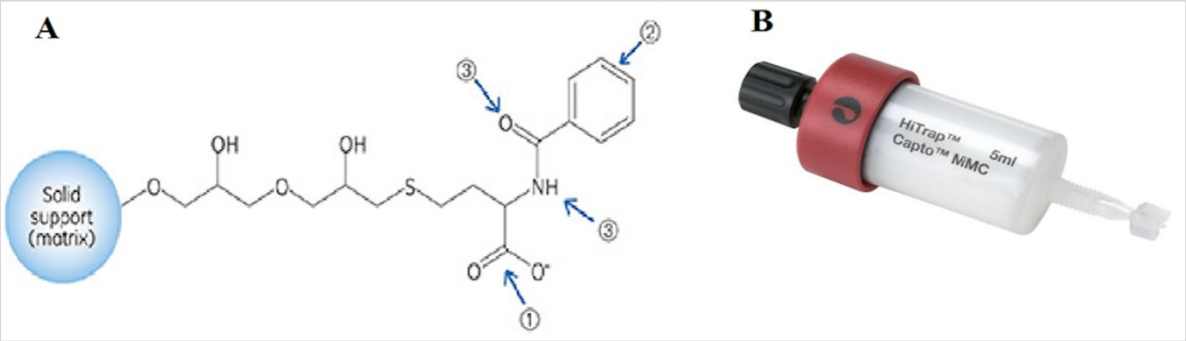


Figure 5: Capto MMC HiTrap, resin. (A) The ligands and its structure (B) Overview of chromatographic column. The possible ligand interactions are (1) Ionic, (2) Hydrophobic interaction and (3) Hydrogen bonding interaction (Y. Yang & Geng, 2011).

2.4 Methods flow-chart

An overview of the experimental work is presented in the following flow-chart (figure 6) which includes the inoculum preparation and bacterial growth, fermentation optimization, enzyme capture and chromatographic separation with supporting analysis for detection of enzyme activity.

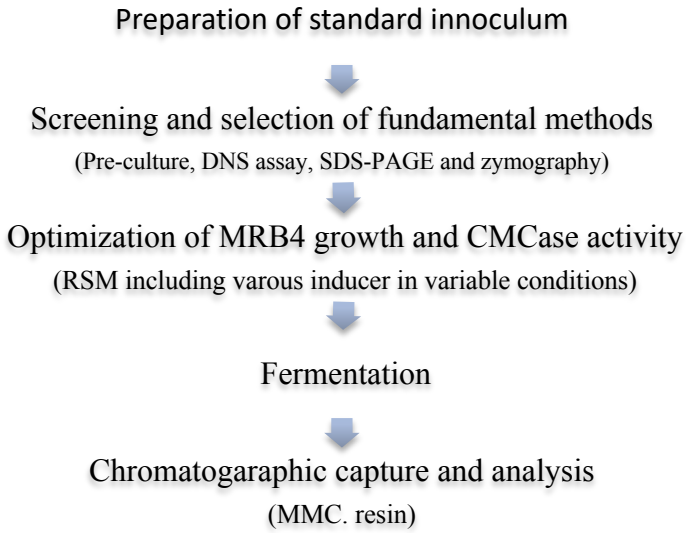


Figure 6: The methodology of the experimental work including all steps for screening of growth conditions, CMCase activity and chromatographic capture.

2.5 Methods

2.5.1 CMCase activity assay

Endoglucolytic activity was measured by the 3, 5 dinitrosalicylic acid (DNS) method using carboxy methyl cellulose to detect hydrolysis of ß-1,4 glycoside bond between glucose units (Ghose, 1987). The subsequent formation of reducing ends (e.g. aldehyde group of reducing ends) is detected by oxidation of one aldehyde group and reduction of 3, 5-dinitrosalycilic acid simultaneously (Miler et al,.1960). The free aldehyde groups i.e. sugar reducing ends are oxidized to carboxylic group, in the presence of oxidizing agent 3, 5-dinitrosalicylic acid (DNS) reagent to 3-amino-5-nitro salicylic acid (reducing agent or coloured product) in alkaline medium. (Ghose, 1987). The orang coloured product is diluted and measured by spectrophotometer at 540nm (Spectroquant@ pharo, 300M).

CMCase activity was measured by incubating 0.5 ml 2% w/v CMC (prepared in 50mM Sodium citrate buffer, pH 7.5) with the reference cellulase enzyme (*Trichoderma reesei* ATCC26921) as positive control and with MRB4 culture sample as test at 50 °C for 30 minutes. The enzyme reaction was halted by adding 3 ml DNS and placed in boiling water bath for 5 minutes then cooled on ice container before adding 20 ml distilled water into each test tube. The amount of reducing sugar was determined by measuring the absorbance at

540nm (Miller, Blum, Glennon, & Burton, 1960). CMCase activity of enzyme was quantified (U ml^{-1}) by using glucose standard curve (0 to1 mg ml^{-1}).

One unit of CMCase activity was defined as the amount of enzyme that released 1µmol reducing sugar (glucose) per minute under standard assay condition. The enzyme activity was measured in unit per millilitre (U ml^{-1}), where one unit was equivalent to µmol $minute^{-1}$. A glucose standard curve was prepared by serial dilution of 1 mg glucose per 0.5 ml glucose in citrate buffer to make different glucose concentrations of 0.25, 0.50, 0.67, and 1.0. After incubation of CMC substrate with diluted glucose samples at 50 °C for 30 minutes 3 ml DNS reagent was added to boil in vigorously boiling water for 5 minutes. The boiled content was diluted with 20 ml of distilled water and the absorbance was measured at 540nm.

A glucose calibration curve was plotted as absorbance of glucose with respect to concentration and used for the quantification of CMCase activity of the sample enzyme under standard assay condition. The equation was obtained by linear regression of calibration curve was:

$$y = 0.924x - 0.0723 \quad \text{eq. (1)}$$

where y denotes the value of absorbance and x denotes glucose concentration. Then CMCase activity was calculated by using the formula:

$$\text{CMCase activity} = (\text{Glucose from standard curve}) / (0.37 \text{ U ml}^{-1} \quad \text{eq. (2)}$$

Where, one unit was equivalent to one µmole $minutes^{-1}$ (Unit = µmole $minutes^{-1}$)

2.5.2 Cultivation of cellulase-secreting bacteria

Shaking culture: For cellulase production, 1ml of MRB4 strains (lyophilized) was cultivated in 250 ml baffled bottle with 150 ml (ABB) Anaerobic Basal Broth (pH 6.8 ± 2 at room temperature) media supplemented with 0.75 gram cellobiose (final concentration 0.5 % w/v), incubated under shaking at 150 rpm on the Infors HT Labotron shaker at 30°C for 24 hours. During incubation, 2 ml samples were withdrawn by sterile pipettes at every 3 hours intervals, for measuring of OD_{600} and CMCase activity until 12 hours of cultivation. A total of six samples: 5 samples were collected in 3 hours intervals, while the final sample was collected after 24 hours incubation. After incubation, the cells were pelleted by centrifugation at room temperature in 2,000 rpm (613 x g) for 5 minutes to obtain a cell free supernatant containing crude enzyme. The culture broth was centrifuged at 4,000 rpm (JA 20 rotor) in a Backman

J2–HS centrifuge for 15 minutes at 25 °C. The clarified crude supernatant was used for screening of enzyme activity by DNS methods as well as further analysis by SDS-PAGE and zymography.

Bench-scale fermentation: For scale up a 3.7 L stirred tank fermentor (Bioengineering AG, Wald, Switzerland) was used, connected with pH control, oxygen probe and temperature control. The fermentor contained 2 L of ABB media without any supplement, sterilized in situ. An overnight pre-culture (150 ml) of MRB4 was added and cultivated under similar condition as established the shaking bottle culture. The fermentor was aerated by supplying sterile air at 200 L h^{-1}, stirring kept at 800 rpm and temperature at 30 °C. After approximating 4 hours of running, the fermentor was supplemented with 20 % cellobiose (10 grams in 50 ml) to final concentration 0.5 % (w/v). Samples approximately 2 ml for OD_{600} and CMCase activity were withdrawn aseptically by siphoning through a silicon tube. Out of 12 samples, the 1st to 11th samples were collected at one-hour intervals, while the 12th sample was collected after 24 hours incubation. After 24 hours, the culture (around 1.8 L) was harvested and stored frozen at -20 °C. About 300 ml was centrifuged at 2,000 rpm for 10 minutes, then 20 ml clarified supernatant was concentrated in a dialysis bag (Spectra/pro® dialysis membrane, 9200820, MWCO: 6-8 KD) covered with solid PEG 8,000. The 20 ml supernatant was finally concentrated to 2 ml for 6 hours and the concentrate carefully removed. The concentrate was analysed by SDS-PAGE for detection of Protein bands (Protein mass) by Coomassie staining, as well as for enzyme activity by zymography using CMC as substrate.

2.5.3 SDS-PAGE and zymography.

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) was carried out according to (Laemmli, 1970), and used to separate proteins according to their molecular weight and unified charges. Staking gel was 3 % polyacrylamide and separating gel was either made in 10 % or 12 % polyacrylamide. The SDS detergent was used in sample buffer to denature the protein structure from tertiary and secondary to primary which also confers even distribution of negative charges to the protein samples. The buffer system used to prepare running gel was 0.5M Tris HCl with 0.15 % SDS, pH 6.8, and for separating gel 1.5M Tris HCl with 0.15 % SDS, pH 8.8.

The positive controls cellulase from *Trichoderma reesei* ATCC26921 and Novozymes NS-81210 were diluted in water 40X and 20X, respectively, prior to mixing with 2X sample buffer

in equal volume (30 µl of each). Controls and samples were heated at 95 ºC not more than 45 seconds to minimize irreversible denaturation, then rapidly cooled on ice container before loading 15 µl samples into the respective gel wells in parallel gels. Gel electrophoresis was run at 180V for approximately 45 minutes with 2X running buffer. After electrophoresis, the protein gels were stained with CBB-250 for one hour, and destined in about 400 ml distilled water, while heated in microwave oven until obtaining clear bands on gels. The gels were viewed by using Kodak image station 4000 MM and the Protein gel viewer software connected with image station. The coloured prestained protein standard stain (11-245 kDa, P7712) was used to compare any size of visible sample bands that appeared on the gels.

In the paralleled analysed gel, CMCase activity of the concentrated fermentor sample was detected by zymography using the method of (van Dyk, Sakka, Sakka, & Pletschke, 2010). CMC (1 %) was incorporated as substrate when casting the separating polyacrylamide gel. Due to CMCase activity, CMC is degraded and detected as bands of clear zones (halo) on a red-purple background, when stained with Congo red, which otherwise intercalate with the undigested CMC.

Proceeding directly after electrophoresis, the zymography gel was soaked in the in the renaturing buffer (2.5 % Triton X-100, 625 µl in 25 ml buffer); 50mM sodium acetate buffer, pH 5.5 for the CMC, and 50mM Tris HCl, pH 7.5 buffer for xylan as substrate. Renaturation was with shaking (60 rpm) for 60-90 minutes. Then, the renatured gel was incubated into the same reaction buffer used in the gel renaturation, but without 2.5 % Triton X-100 detergent, for 24-48 hours at 37 ºC under gentle shaking at 120 rpm. After discarding the buffer, the gel was stained with 0.3 % Congo red for 15 minutes and distained in 1M NaCl until halo bands appeared. As counter stained 0.5 % acetic acid was used. Eventually, the CBB-250 stained protein gel and the zymography gel were aligned together and photographed under white light box to compare and identify active protein bands.

2.5.4 Response surface methodology (RSM)

The individual factors that affects the growth of cellulolytic bacteria were investigated through RSM, also known as Design of Experiments (DoE), selecting factors that are associated with optimal bacterial growth and CMCase activity (Shajahan, Moorthy, Sivakumar, & Selvakumar, 2017). The experiments in the study was designed to optimize the bacterial growth and to detect CMCase activity by including three parameters at two levels; temperature

(30 ºC or 40 ºC), 0 and 2 % w/v CMC and 0 and 25 mg ml^{-1} $CaCl_2$. All three parameters were tested in two different ABB and LB (Luria Bretani) media with 3 ml L^{-1} Tween-20 (surfactant) as default supplement for both media. The total number of experimental runs were defined with the factorial fraction design formula, 2^{n-1}. where, `n` indicates the number factors (variables) either in high or low levels. Fractional design was selected over full factorial (2^n) for the sake of simplicity.

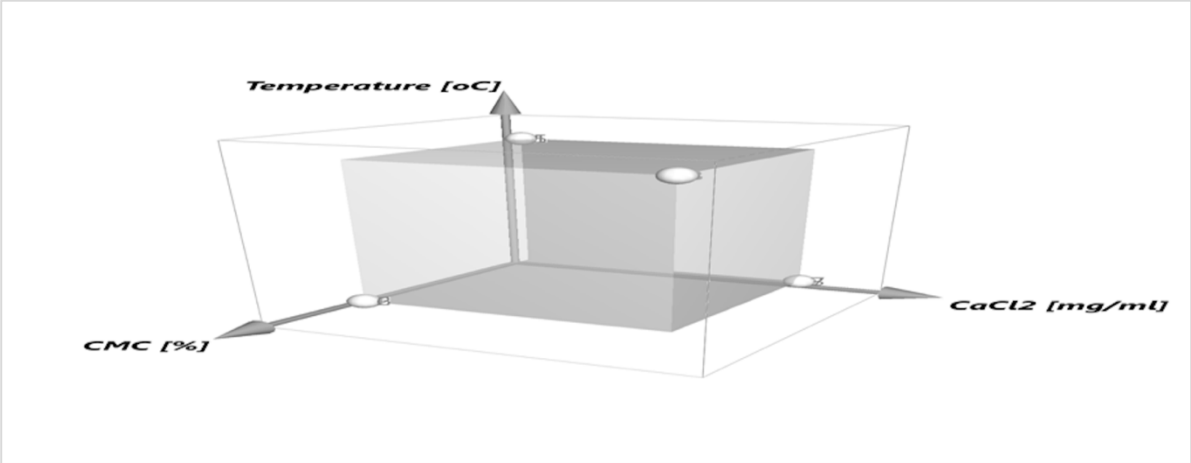


Figure 7: Representation of the design regions of the experiments where four regions are indicated as white dots located in four distant corners.

The experimental design setup including the three variables (n=3), was as shown table 1, but using only four out of eight possible, unique combination (figure 8), e.g. $2^{3-1} = 4$ experiments. The four corners are positioned as distant as possible in the design space. For each of the two media ABB and LB, two cultivation replicates were carried out. Thus, 16 cultivation experiments were carried out; 4 x 2 x 2 = 16.

Table 1. The experimental set up design with four parameters in ABB media and LB media, where -1 represent minimum value and +1 represent maximum value.

Variable details			Coded values	
Variable	Unit	Symbols	–1 level	+1 level
CMC	% w/v	X_1	0	2
$CaCl_2$	Mg L^{-1}	X_2	0	25
Temperature	°C	X_3	30	40
Tween-20	mL L^{-1}	X_4	3	3

The experiments were carried out in shake baffled bottle cultures of 100 ml. As response factors were used OD_{600} measured at 6 hrs. and 24 hrs., and CMCase activity of culture supernatant at 6 and 24 hours measured by assay absorbance at 540nm.

The modelling of the variables used in this experiment was based on a regression polynomial equation generated by the RSM software (MODDE Pro, MKS U metrics, Umeå, Sweden):

$$y = \beta o + \beta 1\chi 1 + \beta 2\chi 2 + \beta 3\chi 3 + \cdots\cdots\cdots . + \varepsilon \qquad eq.(3)$$

where, y = response value (CMCase, OD_{600}), βo = constant variable, βi $(\beta 1, \beta 2, \beta 3, ..)$ = empirical regression coefficients (the main effects) of each variable Xi, $(X1, X2, X3)$ = test variables and ε is the residual term.

The research hypothesis is βi ≠ 0, i.e. the variables will have an effect. The residual term epsilon is not assigned any numerical value but contains any variation in the model not explained by the observations. The parameters for both bacterial growth and the CMCase activity was evaluated from using effect plot and response contour plot. The degree of variance or level of significance of the response surface model was evaluated by ANOVA test. Data analysis and output diagrams were provided by prof. KO Strætkvern.

2.5.6 Stastistical analysis

The analysis of variance is calculated by one-way ANOVA test method where statistical difference at $p<0.05$ was considered significant. As software was used either the MODDE Pro software or the statistical tools of Microsoft Excel.

2.5.7 Protein concentration by ammonium sulphate precipitation

The 24 hours crude fermentation broth (500 ml) kept in freezer at -80 °C was thawed gradually by keeping overnight at 4 °C in the refrigerator. The thawed sample was clarified by centrifugation at 7,000 rpm (7520 x g) (JA 14 rotor) for 20 minutes at 4 °C in a Beckman J2-HS centrifuge. The clarified supernatant was collected by decanting and protein precipitated by ammonium sulfate (AS). To the liquid was gradually added AS until 80 % saturation. The amount of salt (grams of AS required for per ml) was calculated using the formula;

$$\text{Gram (g)} = \frac{533(S2-S1)}{100-0.3\ S2} \cdots\cdots\cdots\cdots\ eq\ (4)$$

where, S2 was the maximum saturation (80 %) and S1was the minimum saturation (0 %)

The AS saturated sample was cooled on ice for 30 minutes, then centrifuged at 10,000 rpm for 30 minutes obtained protein pellets. The 2/3rd part of supernatant was discarded leaving the sediment which was then dissolved with 0.025 M sodium acetate, pH 4.5 (column buffer A) and collected into the volume of 400 ml. The conductivity (Ω = mS cm^{-1}) of the re-dissolved pellet was 118.8 mS cm^{-1}. Then the sample was serially diluted into 1:2, 1:4, 1:8, 1:16 and 1:32 with distilled water to decrease the conductivity to 61.6, 34.1, 18.7, 10 mS cm^{-1} and 5.1 mS cm^{-1} respectively. The diluted sample volumes were 100 ml and used in mixed mood chromatography (MMC).

2.5.8 Capture and fractionation of CMCase by MMC

All the prepared solutions required for the protein chromatography such as, start buffer A (0.025 M sodium-acetate, pH 4.5), elution buffer B (0.025M phosphate buffer, 1M NaCl, pH 7.7) and serial diluted samples were filtered with Glass microfiber filters (pore size 1.2πm, Whatman®) applying vacuum suction.

The AS concentrated, and serially diluted samples were chromatographed on the ÄKTA Start chromatography system with Frac 30 fraction collection and Unicorn™ start 1.1 software for the full control of operation (GE Biosciences, Uppsala, Sweden). A 5 ml HiTrap multimodal column (HiTrap Capto MMC, 5 ml) was supplied by GE health care used for the chromatography separation of the sample.

Capto MMC ligand acts through multiple interactions such as, ionic, hydrogen bonding and hydrophobic in order to capture the protein molecules. (Kallberg, Johansson, & Bulow, 2012). All the serial diluted samples with different conductivity was used as sample and feed into the instrument by automatic suction method together with start buffer (0.025M Na-acetate, pH 4.5). The flow rate into the column was 5 ml min^{-1}. The unbounded protein was washed out with 8 ml start buffer A. The gradient size was 5 column volumes (cv, e.g. 25 ml) of 0-100 % buffer B, followed by 2 cv buffer B. Buffer B was used for the elution of the captured protein and the eluted fractions (8 ml) were collected. Protein was monitored in-line as absorbance at 280 nm (UV 280) and the salt gradient monitored by an in-line conductivity sensor.

3. RESULTS

3.1 Cultivation of MRB4 on ABB media

The MRB4 bacterial isolates from moose rumen was cultivated at 30 °C in 250 ml baffled flask with 150 ml ABB media where two replicates were supplemented with 0.5 % w/v (0.75 gram) potato rasp and two without. One of each was with shaking (aerobic conditions, 150 rpm) another without shaking for 24 hours. Four growth curves were obtained (Figure 8). The lag-phase for MRB4 cultivated with rasp, but without was approximately 6 hours, while with rasp and shaking growth was observed after 3 hours and ended within 6 hours in ABB media. The growth curves for MRB4 in ABB without rasp were similar for most of the 24 hours of cultivation. Thus, shaking condition and in presence of rasp, the lag phase was brief, and growth was most rapid, and reached a maximum level after around 9 hours. In general, rest of the curves showed nearly similar logarithmic growth curve phase that started after 6 hours and remain until around 12 hours then proceed to the stationary phase in all growth conditions. Rasp was used as a probable inducer of growth and CMCase activity (Rabiul, 2016). The cell densities did not increase significantly either in presence or absence of rasp in ABB media as supplements and was not used further in the experiments.

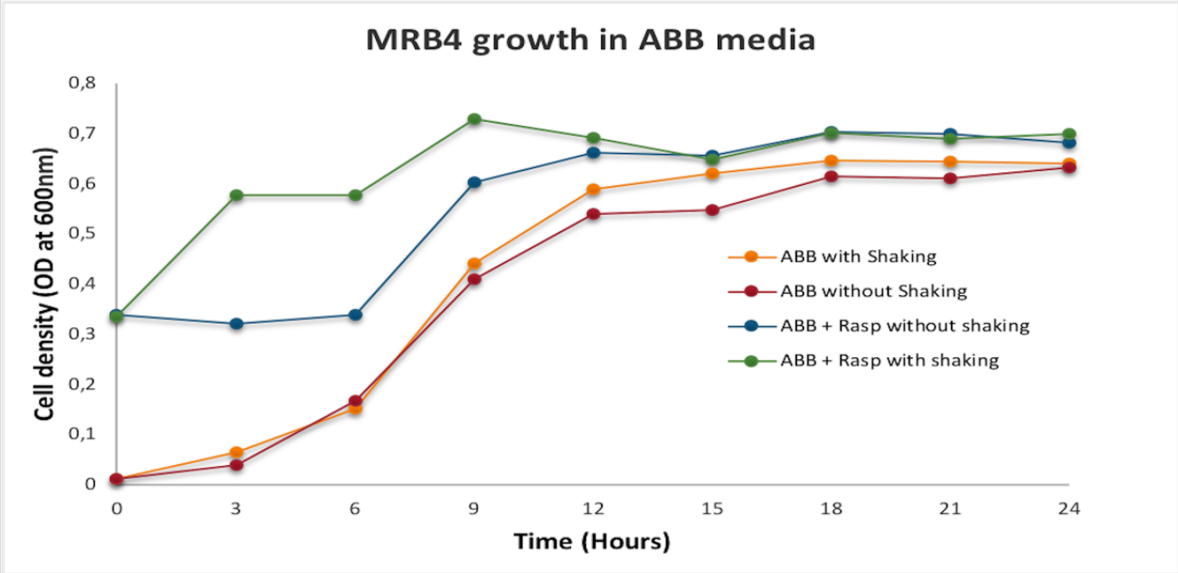


Figure 8: Growth curves of MRB4 isolate cultivated at 30 °C under aerobic condition in ABB media supplemented with 0.5 % w/v potato rasp with or without shaking.

The growth curves in figure 8 shows the growth curves of MRB4 isolate, grow on the ABB media with 0.5 % w/v rasp as carbon source. The cultures were monitored for 24 hours, but

the cell densities were not increase significantly either in presence or absence of rasp in ABB media as supplements.

3.2 Potato juice (PJ) as MRB_4 culture media

The MRB4 was cultivated at different temperature for 24 hours in autoclaved potato juice (15 ml PJ), supplemented with 750 µl of 20 % w/v stock glucose and 0.5 % w/v (75 mg) rasp (Figure 10). Cellulose/hemicellulose holding rasp is suggested as a simple inducer for cellulase production in the MRB isolates (Rabuil, 2016).

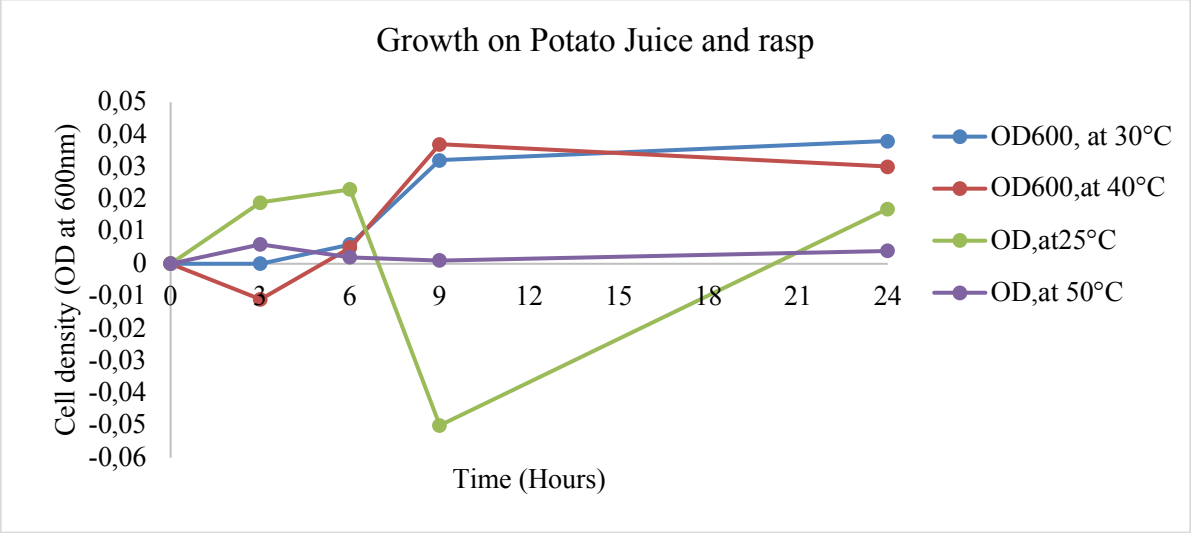


Figure 9: MRB4 culture in 15 ml PJ in presence of potato rasp (1 %) and glucose supplement under variable temperature conditions. Constant shaking of glass tubes (20 ml, 16 mm) at 150 rpm.

The growth in PJ showed modest but positive response at 30 °C and 40 °C in presence of growth supplements (1 % w/v) glucose and 0.5 % w/v potato rasp), but less growth at room temperature (25 °C). The cell density at 50 °C was around zero (remained unchanged) for entire growth period (24 hours). From the cultivation curves, 30 °C and 40 °C were considered the best growth temperatures for MRB4, and 30 °C selected for further experiments. However, PJ supported very low growth even when including potato rasp and glucose as supplement. Potato juice was not pursued further as growth media.

3.4 Growth and endoglucanase activity on ABB media

The MRB_4 isolate was cultured under shaking at 30 °C for 24 hours in 150 ml ABB media supplemented with both 0.5 % w/v cellobiose and 0.5 % w/v potato rasp as enzyme inducers.

The samples were withdrawn at every 3 hours intervals up to 24 hours in order to determine CMCase activity and the bacterial cell density. The progress of growth and the CMCase activity is shown in the figure 10.

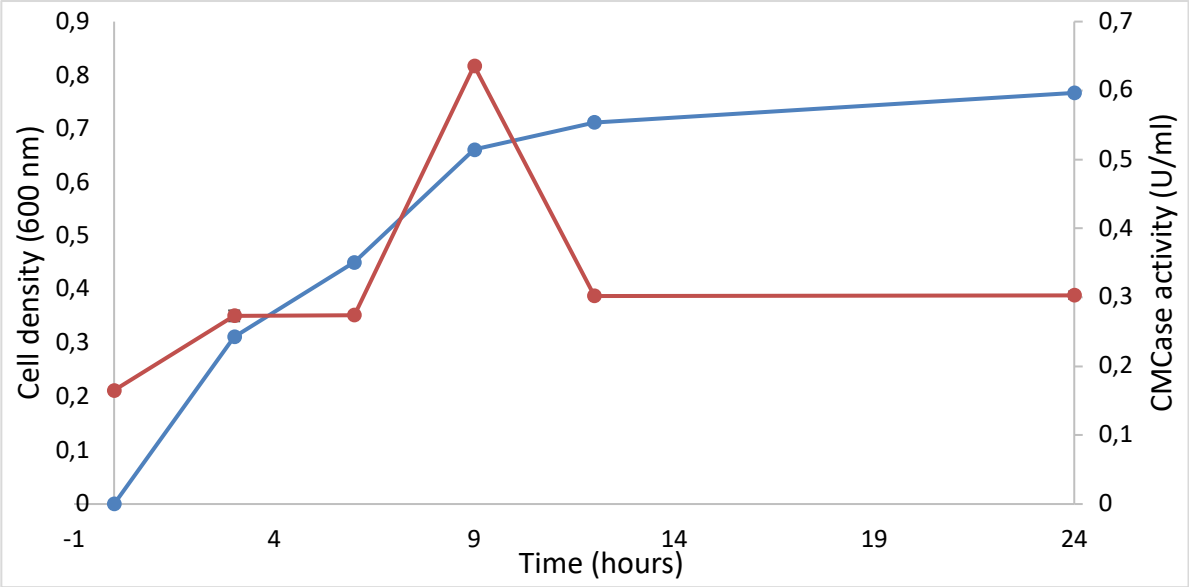


Figure 10: Growth (OD $_{600}$, blue line) and CMCase activity curve (red line) obtained by cultivation of MRB4 in ABB media (150 ml) supplemented with cellobiose (0.5 %) and rasp (0.5 %). Temperature was at 30 °C and rotary shaking at 120 rpm. The CMCase activity was measured in replicate and the error bars was seen very small.

The cultivation of MRB4 supplemented with 0.5 % cellobiose and 0.5 % rasp showed positive effects on both bacterial growth and enzyme activity (Figure 10). Cellobiose not only had background effects on CMCase activity measurements as it started from about 0.166 U ml^{-1} at zero hours, but it also initiated early starting of the log phase extended approximately for 10 hours. Cultivation with cellobiose and rasp resulted maximum CMCase activity at around 9 hours (0.635 U ml^{-1}) though highest bacterial cell density was at around 12 hours and at 24 hours which was (0.712 OD_{600} unit and 0.767 OD_{600} units respectively). The CMCase activity declined gradually after 10 hours of incubation and the rate of dropped down was around two folds at 24 hours sample than 9 hours sample. The progress of bacterial growth and the CMCase activity curve indicated that the culture could be harvested for highest enzymatic yield at around 9 hours.

3.5 Growth and induced enzyme activity of MRB_4 in ABB by bench scale fermentation

After having tested growth temperature, growth media and supplements in small scale, bench scale fermentation of MRB4 was carried out in 2.0 litter working volume of ABB media. This was to obtain increased enzyme yield under controlled supplementation i.e. induction with cellobiose. The samples were withdrawn at every half hours interval up to 11 hours to measure CMCase activity and bacterial OD_{600} and the final measurement of the overnight culture (24 hrs.). The cellobiose was added as inducer after 3.6 hours and showed a significant positive effect enzyme activity level rising from ca 0.18 to 0.30-0.35 U ml^{-1}. The OD_{600}, however decreased gradually and reached 0.435 OD_{600} unit after 24 hours with corresponding CMCase activity at 0.34 U ml^{-1}.

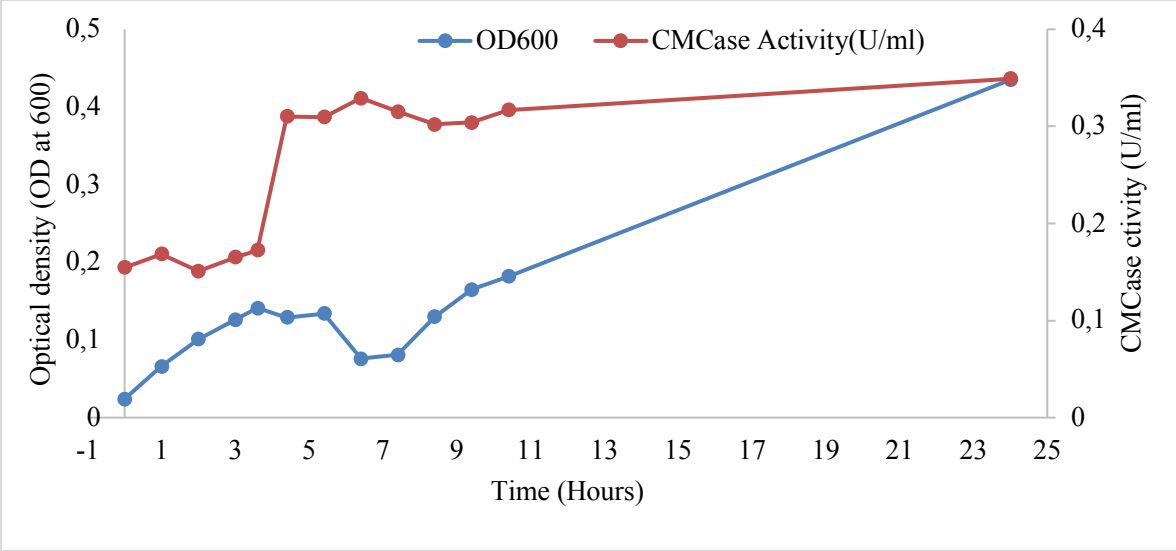


Figure 11: The growth and CMCase activity in bench scale fermentation of MRB4 of ABB media (2.0 L) at 30 °C supplemented with cellobiose (0.5 %) as an inducer.

The CMCase activity curve obtained by cultivation 150 ml of ABB from start with 0.5 % cellobiose (figure 10) and the CMCase activity curve in the bench scale fermentation (2.0 L) were more or less similar. In both curves, the maximum enzyme activity was found after 6 to 9 hours of cultivation. But the growth curves were quite different; highest optical density was found during 6 to 9 hours for the 150 ml culture, while in the fermentor, optical density decreased to minimum level (Figure 11). At the same time period, the OD_{600} is about 6 fold lower than in figure 10, even though both cultures had approximate and the similar enzyme activity level. This temporary decrease may be due to an extended lag phase of bacterial growth which is may be due to cell lysis and death.

3.6 SDS-PAGE and zymography

Figure 12 shows the SDS-PAGE analysis of PEG-concentrated 24 hours culture supernatant compared with reference cellulase enzyme. The zymogram stained with Congo red (figure 12 A), shows the enzyme active halo bands appear between the 58 kDa band and 46 kDa and there was also a faint halo band on around the 60 kDa. Corresponding protein bands are pointed by black arrows in the Coomassie stained gel (Figure 12 B). The MRB4 cellulase enzyme separated by SDSS-PAGE revealed that cellulolytic bands were at around 52 kDa and at 61 kDa indicated by molecular weight marker as shown in figure 12 A and B. However, sample bands in both gels are weak compared to the diluted reference enzymes.

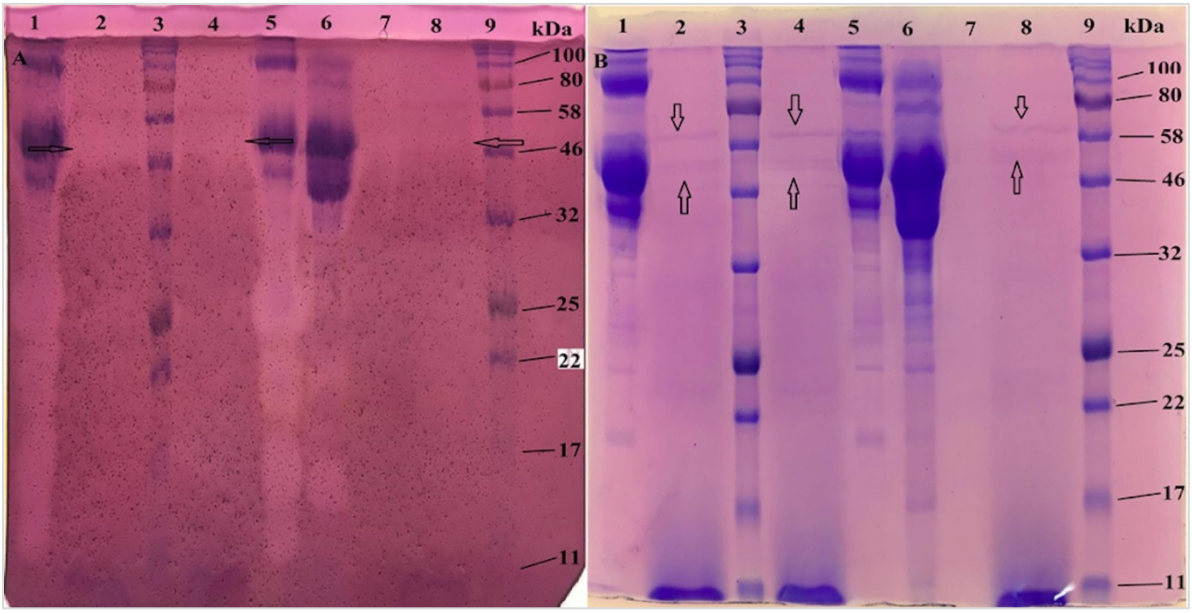


Figure 12: SDS-PAGE analysis and in-gel activity of cellulase enzyme from MRB4. (A) Zymography and (B) Coomassie protein stain. Lane 1: Reference cellulase from *Trichoderma reesei,* 40X diluted, Lane 2: 24 hours reactor sample concentrated with PEG, Lane 3: Stained molecular weight protein marker, Lane 4: 24 hours Reference cellulase and hemicellulase enzyme (DSM), 20X diluted, Lane 7: blank, Lane 8: 24 hours reactor sample, PEG concentration, Lane 9: protein ladder. Black arrow indicates the halo bands representing cellulase enzymes.

The active halo bands in the zymography, both between 52 kDa-61 kDa and with the reference enzymes (several bands) denoted that the cellulase were able to hydrolyse the CMC substrate. The analysis also suggests that the enzymes were sufficiently denatured and properly refolded properly after SDS-PAGE electrophoresis and during the incubation periods.

3.7 Optimize growth through fractional fractorial design

The CMCase activity and OD_{600} were response factors of the MRB4 cultivated in ABB and LB media by using fractional factorial design including 2 % w/v CMC, 25 mg L^{-1} $Cacl_2$ and the temperature at 30 °C and 40 °C. All the experiments contained 3 ml L^{-1} Tween-20.

Table 2: The effects of different variables applied in RSM for cell density and CMCase activity using MRB4 isolates.

ABB media								
Labels	Run no. replicate	Variables			Cell density (600 nm)		CMCase activity (U ml^{-1})	
		X1	X2	X3	6 hrs	24 hrs	6 hrs	24 hrs
1	R1	-1	-1	+1	0.019	0.048	0.368	0.269
5	R2	-1	-1	+1	0.312	0.074	0.401	0.34
2	R3	+1	-1	-1	0.017	0.54	0.331	0.28
6	R4	+1	-1	-1	0.223	0.545	0.32	0.22
3	R5	-1	+1	-1	0.012	0.158	0.411	0.29
7	R6	-1	+1	-1	0.32	0.162	0,38	0.21
4	R7	+1	+1	+1	0.02	0.514	0.521	0.26
8	R8	+1	+1	+1	0.256	0,484	0,441	0.25
LB media								
Labels	Run no. replicate	Variables			Cell density (600 nm)		CMCase activity (U ml^{-1})	
		X1	X2	X3	6 hrs	24 hrs	6 hrs	24 hrs
1	R1	-1	-1	+1	0.013	0.03	0.189	0.2
5	R2	-1	-1	+1	0.016	0.029	0.201	0.185
2	R3	+1	-1	-1	0.016	0.118	0.191	0.041
6	R4	+1	-1	-1	0.019	0.117	0.18	0.032
3	R5	-1	+1	-1	0.031	0.128	0.229	0.027
7	R6	-1	+1	-1	0.42	0.14	0.183	0.034
4	R7	+1	+1	+1	0.52	0.164	0.194	0.036
8	R8	+1	+1	+1	0.57	0.162	0.203	0.034

Figure 13 depicts the outcome of the factorial design for optimizing growth and enzyme yield in MRB4 in ABB. The diagrams provide prediction line for CMC shows that the 2 % CMC substrate had positive influence. Even though including 25 mg L^{-1} $CaCl_2$ and temperature increase discouraged the bacterial growth, both parameters showed positive correlation on CMCase activity (Table 1).

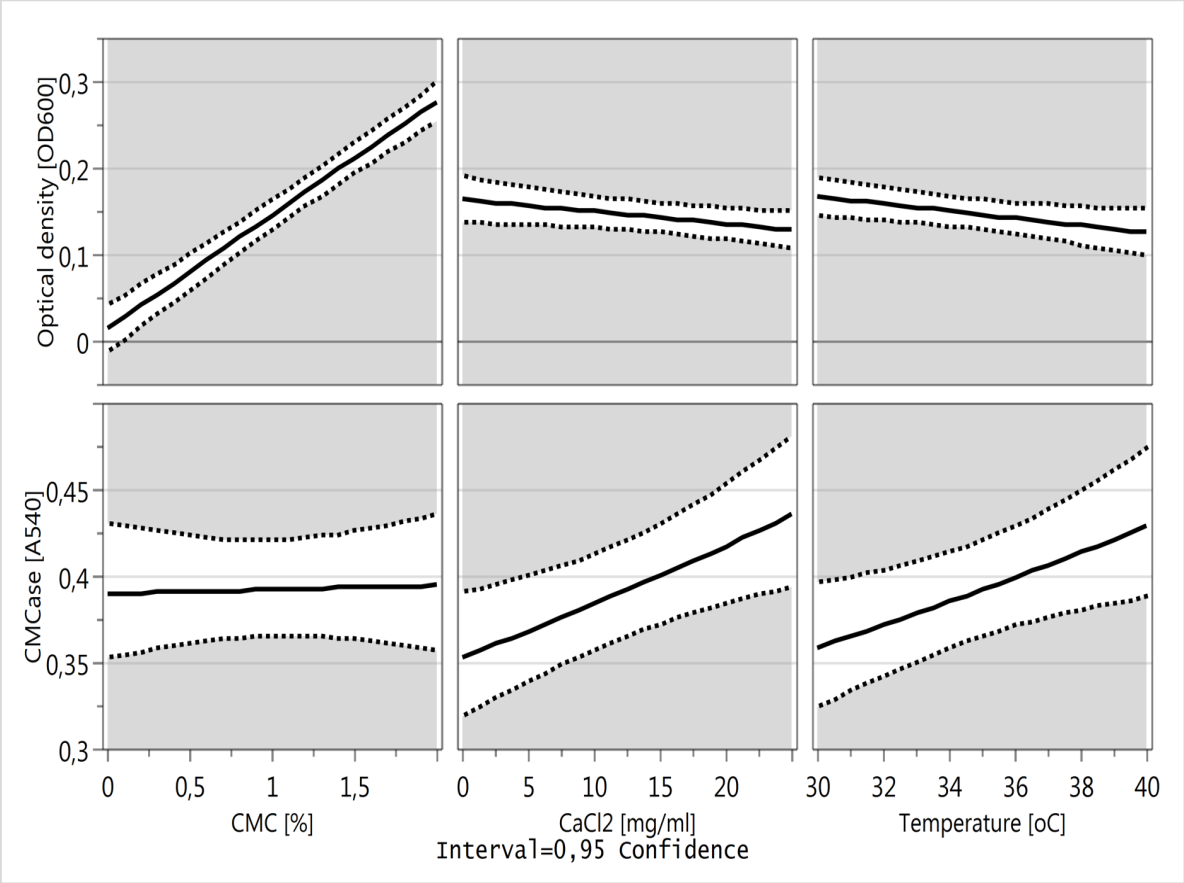
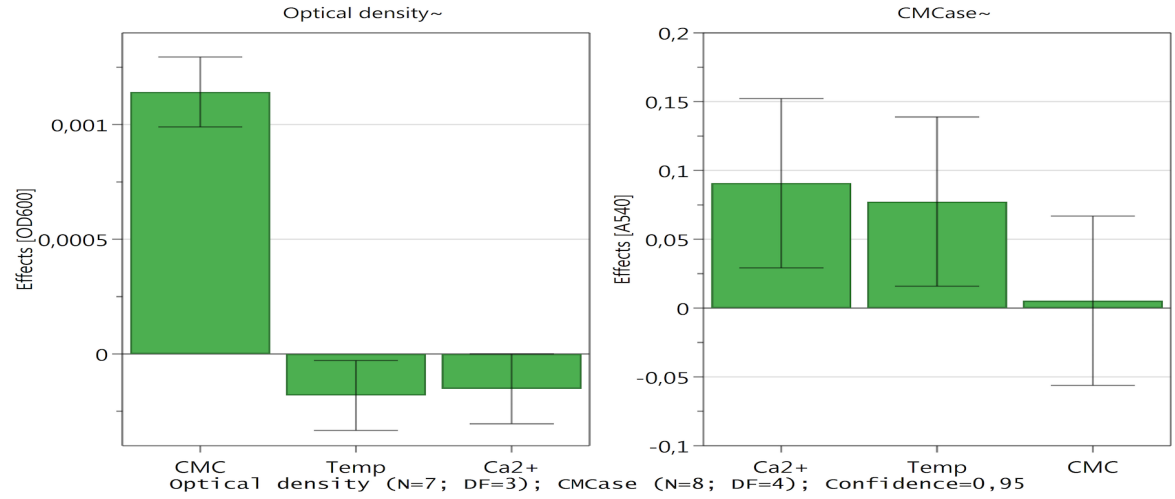


Figure 13. Testing the responses of the most influencing parameters on growth and CMCase secretion of MRB4 in ABB media. The prediction plots illustrate for optimal density and CMCase activity of the MRB4 by using fractional fraction design experiments. The area between dotted lines corresponds to the 95 % confidence interval of each correlation.

The 95 % confidence intervals of OD_{600} in the graphs were closer to each other for all influencing parameters, while for CMCase activity graphs the intervals were wider, indicating lower certainty of the influencing parameters to the CMCase activity.

Effects plots of the parameters tested (figure 14 A and B) show that the two parameters such as $CaCl_2$ and temperature enumerated positive and significant effect on CMCase activity at 6 hours collected samples, but they showed negative and insignificant effects at 24 hours sample.

A



B

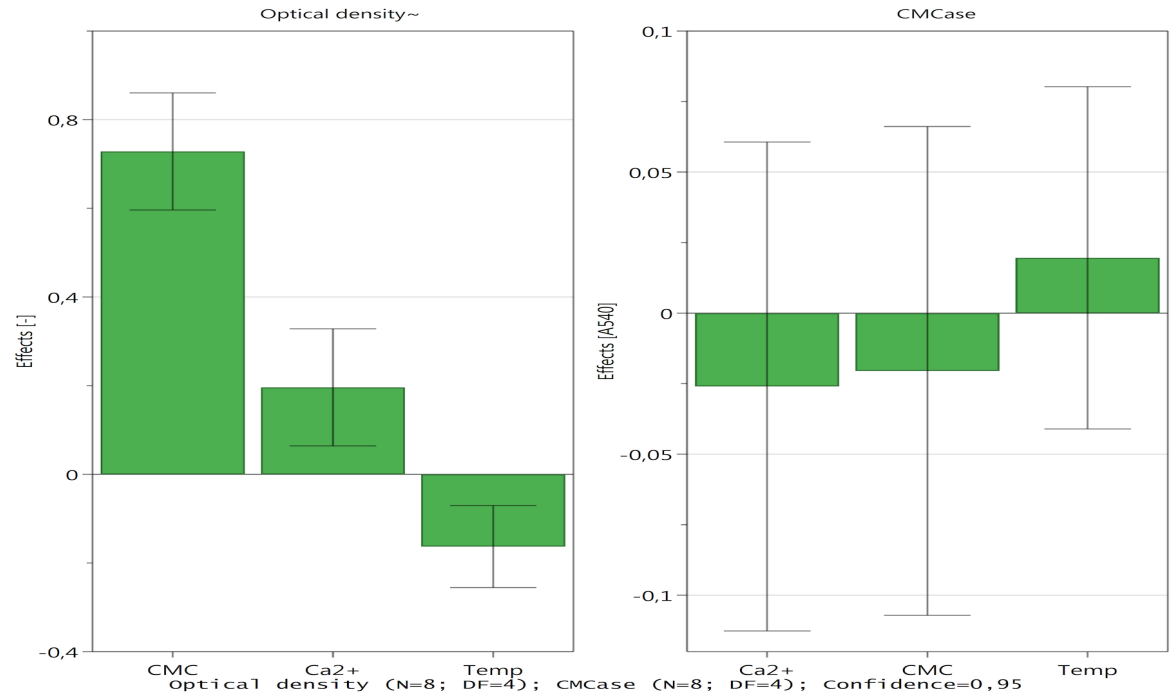


Figure 14: Effect plot obtained by cultivation of MRB4 in ABB media supplemented with 2 % CMC, 25 mg L^{-1} $CaCl_2$ and temperature. Effects of the parameters on cell density and CMCase activities after (A) 6 hours, and (B) 24 hours of incubation.

At 6 hours, the CMC supplement caused a negative effect on CMCase secretion, and at 24 hours non-significant effect as shown by the large span of the error bars (B, right panel). On the other hand, CMC substrate expressed positive and significant effect on cell density in both

6 hours and 24 hours sample. The $CaCl_2$ supplementation showed the positive effect on bacterial OD_{600} at 24 hours samples, but it had negative effect at 6 hours sample (figure 14 A and B, left panels). However, the increased temperature (40 °C) showed negative effects in bacterial cell density in both 6 hours and 24 hours sample, but it had marginal positive effect on CMCase activity.

Two-dimensional contour plots are predictive graphical model based on experimental data, showing the direction of effect of the two most significant parameters. The same coloured strips are isobars for conditions giving the same output. In figure 15, the contour plots for MRB4 in ABB are shown for CMC and $CaCl_2$ at intermediate temperature 35 °C. In ABB media 1.5 % to 2 % CMC substrate was optimal for the MRB4 growth as well as for CMCase activity. Supplementation of $CaCl_2$ (figure 15 A and B) was predicted to have opposite effect on growth (top right corner) and CMCase (bottom right corner), which was also observed from the effect plots (Figure 14 and 15 A` and B`).

Contour plots predicting optimal cell density and CMCase activity of MRB4 (figure 15 C and 15 D) showed that $CaCl_2$ and CMC with concentration around 15 mg ml^{-1} and 1.5 % w/v respectively, can enhanced optimal cell density, while the highest CMCase activity can be obtained during higher $CaCl_2$ concentration (25 mg ml^{-1}), but lower CMC concentration (0.25 %) as shown in the contour plots figure 15 C and 15 D. The effect bar graphs (figure 15 C`) showed that $CaCl_2$, CMC and temperature had positive effects on bacterial growth although the level of CMCase activity was very low. The temperature had the least effect on bacterial growth with larger error bar and considered as statistically insignificant.

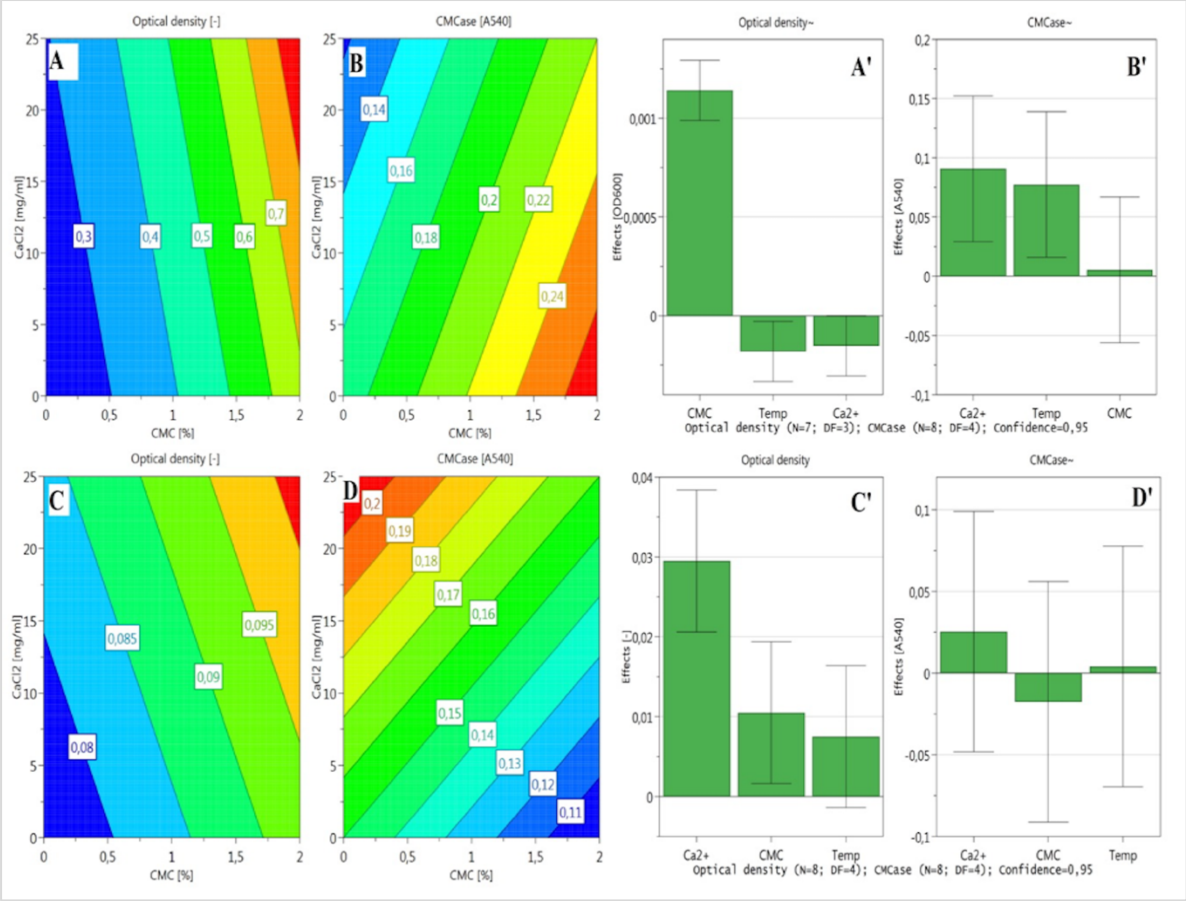


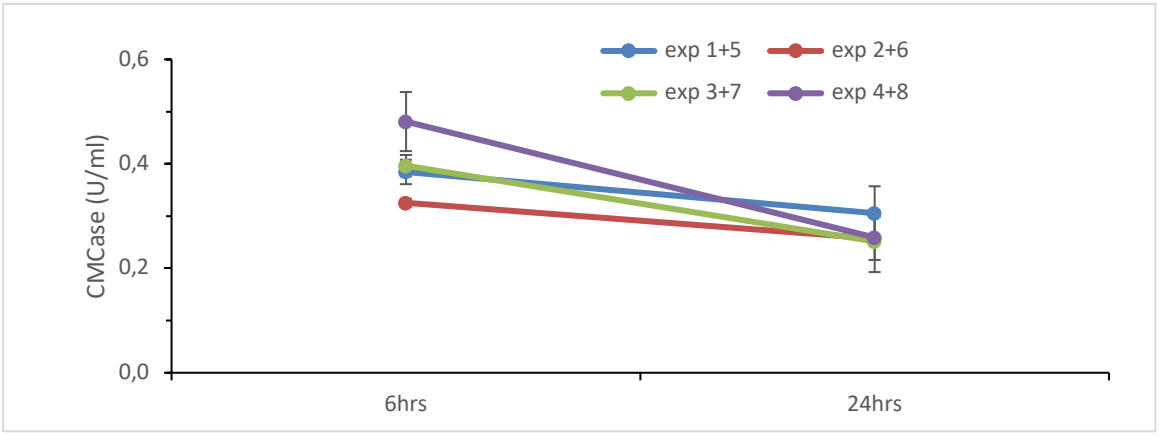
Figure 15: Contour plots and the effects plots showing the mutual and individual effects of CMC substrate, $CaCl_2$ concentration and temperature in optimal bacterial growth and CMCase activity. Figure A, A` and B, B` represent the contour plot and effects plot of 6 hours RSM sample on ABB with their effects respectively. And, figure C, C` and D, D` showed the contour plots and effect plots of 24 hours sample on ABB media with their effect.

All the parameters used for optimization of CMCase activity in LB media yielded low CMCase and effects were considered as statistically insignificant because of larger error bars than the effects bar (Figure 15 D`). Based on contour plot and effect plot, satisfactory growth and CMCase activity was not obtained in LB media with different optimizing parameters and was considered as least supportive for MRB4 growth and CMCase activity. However, the cell density in the 24 hours sample was higher, while the CMCase activity was declined in prolonged culture after 24 hours incubation in ABB media (Figure 15 lower right and left).

3.8 Prolonged culturing and its effect in bacterial cell density and CMCase activity

The outcome of growth and CMCase activity and 6 and 24 hours in the DoE experiment was compared. According to the figure 16, the effect of prolonged culturing on cell density and CMCase activity was different with each other. The cell density was increased around two folds from 6 to 24 hours, while CMCase activity decreased during the same time span. The bacterial cell density increased most markedly in presence of 2 % CMC as supplements Further, the optimal cell density was obtained in presence of 2 % w/v CMC as supplements (exp. 2+6 and 4+8); the cell density was higher at both 6 hours and 24 hours then the cultivation in absence of CMC (exp. 1+5 and 3+7). Thus, addition of CMC and $CaCl_2$ in addition to Tween-20 showed the direct proportional effect in the cell density of prolonged culture.

A



B

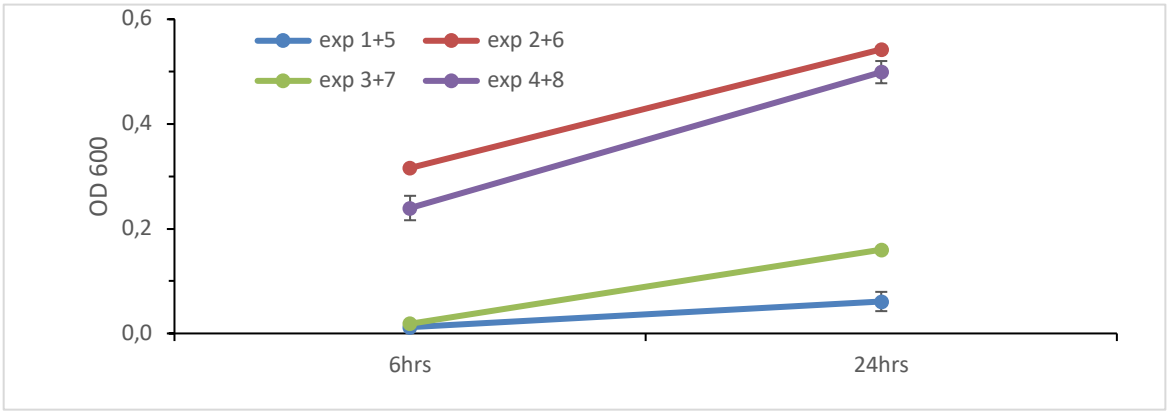


Figure 16: The effect of prolonged culturing on (A) cell density (B) the CMCase activity of MRB4 in ABB media. Data points connected with lines of same color are average of replicate (n = 2). Experiment numbers refer to Table 1.

Unlike the cell density, the CMCase activity was decreased in prolonged culture (Figure 16 B). The CMCase activity was found higher in all samples at around 6 hours than at the end point (24 hours) samples. At 6 hours, the CMCase activity was about 0.4 ± 0.1 U ml^{-1} but declined by two folds after 24 hours (0.2 ± 0.02 U ml^{-1}).

3.9 Effects of various supplement inducer in cell density and CMCase activity of MRB4 in ABB media by bench scale fermentation

The observed effects of inducer (cellobisoe, figure 11) as well as the various supplements (CMC, $CaCl_2$ and Tween-20) to ABB were tested in a second bench scale fermentation (F II). This experiment is shown in figure 17 A and compared with the fermentation F I. In the F II, cellobiose was added at time of 4.28 hours, but in FI it was added at 3.66 hours, which was 0.61 hours later than the F I. The cell density increased gradually with increasing culture time, but the final (24 hrs.) CMCase activity declined with respect to the cell density (Figure 17 B). The initial CMCase activity was around 0.1 U ml^{-1} and raised up four folds (0.4 U ml^{-1}) after addition of the 0.25 % inducer (25 ml from 20 % cellobiose stock).

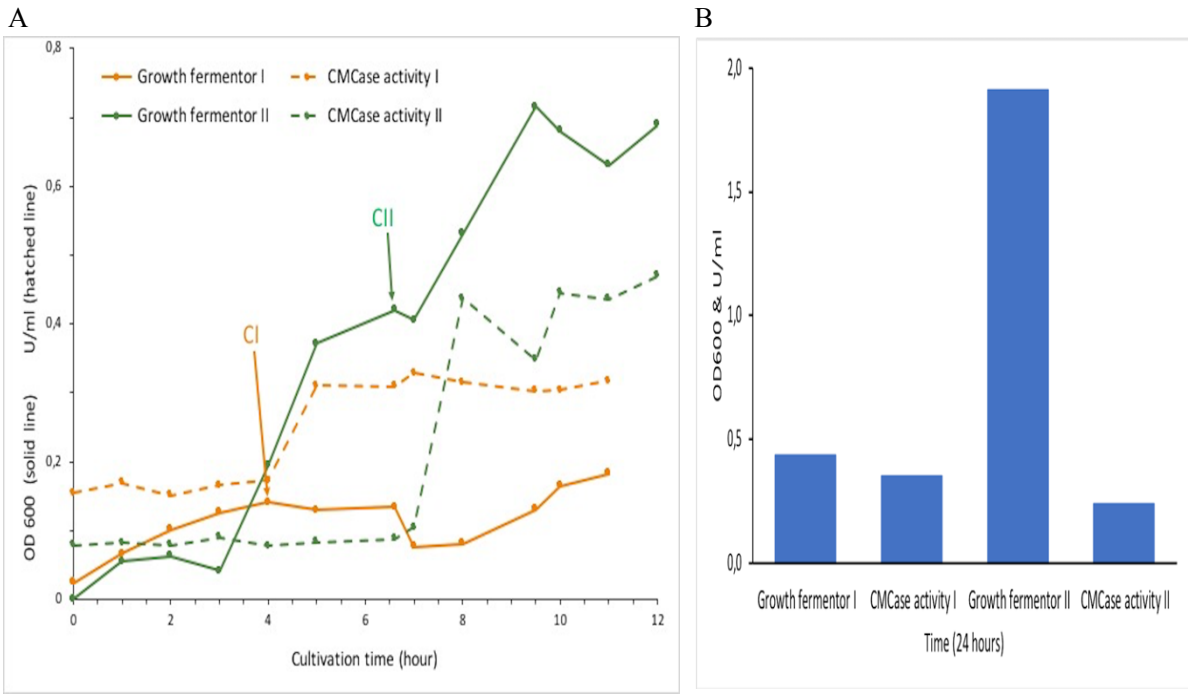


Figure 17: Evaluation of effect of various supplements and inducer in bacterial cell density and CMCaes activity in scale up fermentor. (A) The monitoring of cell density and CMCase activity of two fermentation F I and F II until 12 hours of incubation where the first fermentor was based on outcome of optimization (DoE). (B) Bar graphs representing the 24 hours cell density and CMCase activity. C I and C II indicate the time when the cellobiose added in F I and F II respectively.

The bacterial cell density was increased gradually with few minor fluctuations during the course of growth (Figure 17). The highest cell density after log phase was measured at around 6 to 9 hours of growth and the highest cell density into stationary phase was obtained at 24 hours; OD_{600} 0.435 and 0.91 in F I and F II respectively (Figure 17 B). In F I at zero hours (t = 0), the cell density measure in OD_{600} and CMCase activity was 0.024 and 0.15 U ml^{-1} respectively, while the OD_{600} in F II was tentatively same with the OD_{600} of media blank, considered as zero and the CMCase activity was 0.078 U ml^{-1} which was two folds less than the CMCase activity at zero-hour (Figure 17, Appendix VI). After addition of the 0.5 % w/v and 0.25 % w/v cellobiose in F I and F II at time of 3.66 hours and 4.28 hours respectively, the cell density was decreased in both F I and F II, but the CMCase activity was increased by two folds in F I and four folds in F II. The two folds increases in CMCase values in F I was from 0.18 U ml^{-1} to 0.31 U ml^{-1} and four folds increases in FI was from 0.1 U ml^{-1} to 0.43 U ml^{-1} (appendix VIII).

The OD_{600} was increased gradually and reached 0.435 and 1.91 OD_{600} units in F I and F II after 24 hours respectively. The OD_{600} of F II after 24 hours was 5 folds higher than the OD_{600} of F I. At the time of harvest sample of F II, the CMCase activity had declined by two folds to 0.23 U ml^{-1} from 0.44 U ml^{-1} (at 9 to 12 hours), but in another way CMCase activity at 24 hours, was increased by three folds to 0.23 U ml^{-1} from 0.078 U ml^{-1} at zero-hour. (appendix VIII). Based on bacterial cell density curve and the CMCase activity curve the suitable culture harvesting time was between 6 to 9 hours because of higher cell density and the maximum CMCase activity.

3.10 Screening of binding conditions of CASP: Sample loading, flow rate and conductivity

The CMCase enzyme was purified from the fermentation broth using ammonium sulphate (AS) precipitation and mixed mode chromatography (MMC). The AS-precipitation was carried out by saturation up to 80 % that partially helped to separate it from other proteins, but more important concentrated the enzyme. The precipitated, and subsequently re-dissolved protein pellet was named CASP. In the mixed mode chromatography, the CASP, including any CMCase was adsorbed with HiTrap Capto MMC resin with (5 ml), loading samples with different volumes and dilutions e.g. conductivity. This approach revealed variation as well as

effects in the yield of captured enzyme in eluted fractions. The initial conductivity of the CASP solution was 111.8 mS cm^{-1}(pH 4.7), which was then diluted serially to 3X, 5X, 9X, 17X and 33X aliquots of diluted samples to 61.6, 34.1, 18.7, 10.0, and 5.1 mS cm^{-1} respectively (Appendix VII).

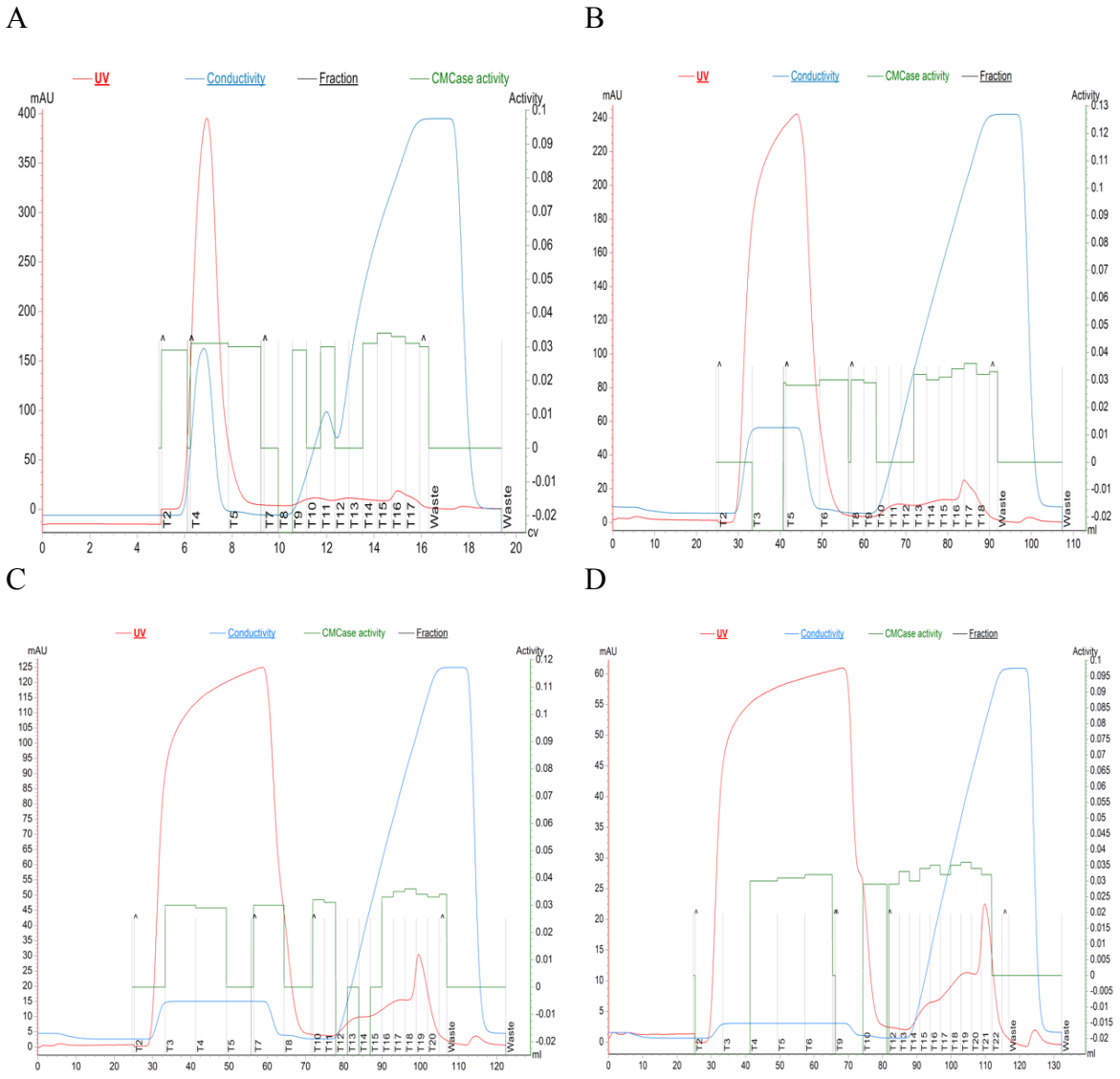


Figure 18: Mixed mode chromatography of dissolved AS pellet of fermentation broth with different sample loading volumes, flow rates and conductivity. The column loading volume was increased with decreasing conductivity of samples to load approximately same amount of protein. In (A) load was 5 ml sample of 5X (34.1 mS cm^{-1}); in (B) 15 ml of 9X (18.7 mS cm^{-1}); in (C) 30 ml of 17X (10 mS cm^{-1}), while in (D) 40 ml of sample of 33X (5.1 mS cm^{-1}) diluted CASP was loaded. The green bar graphs indicate CMCase activity level (U ml^{-1}) in individual fractions. Blue line is conductivity and red line is the protein UV trace at 280 nm.

Different levels of sample loading, flow rate, and conductivity were tested to find out the variation in the CASP yield. To load comparatively same amount of protein sample volume increased proportionally with higher dilution. In figure 18, the chromatograms (A, B, C and D) depict longer and lower flow through peaks, usually denoted by the fractions T2 to T10, as the sample load become higher.

In figure 18 A, the chromatogram, the sample loading volume was 5 ml with 31.1 mS cm^{-1} conductivity, flow through fraction were T2 to T5, while the flow through size become gradually longer in the figure 18 B (fraction T2 to T6), 18 C (fractions T2 to T8), and 18 D (fractions T2 to T10) due to increase sample loading volume from 5 ml, 15 ml, 30 ml and 40 ml respectively. The major elution peaks were similar in all chromatograms though small variation in size and shape according to the sample conductivity.

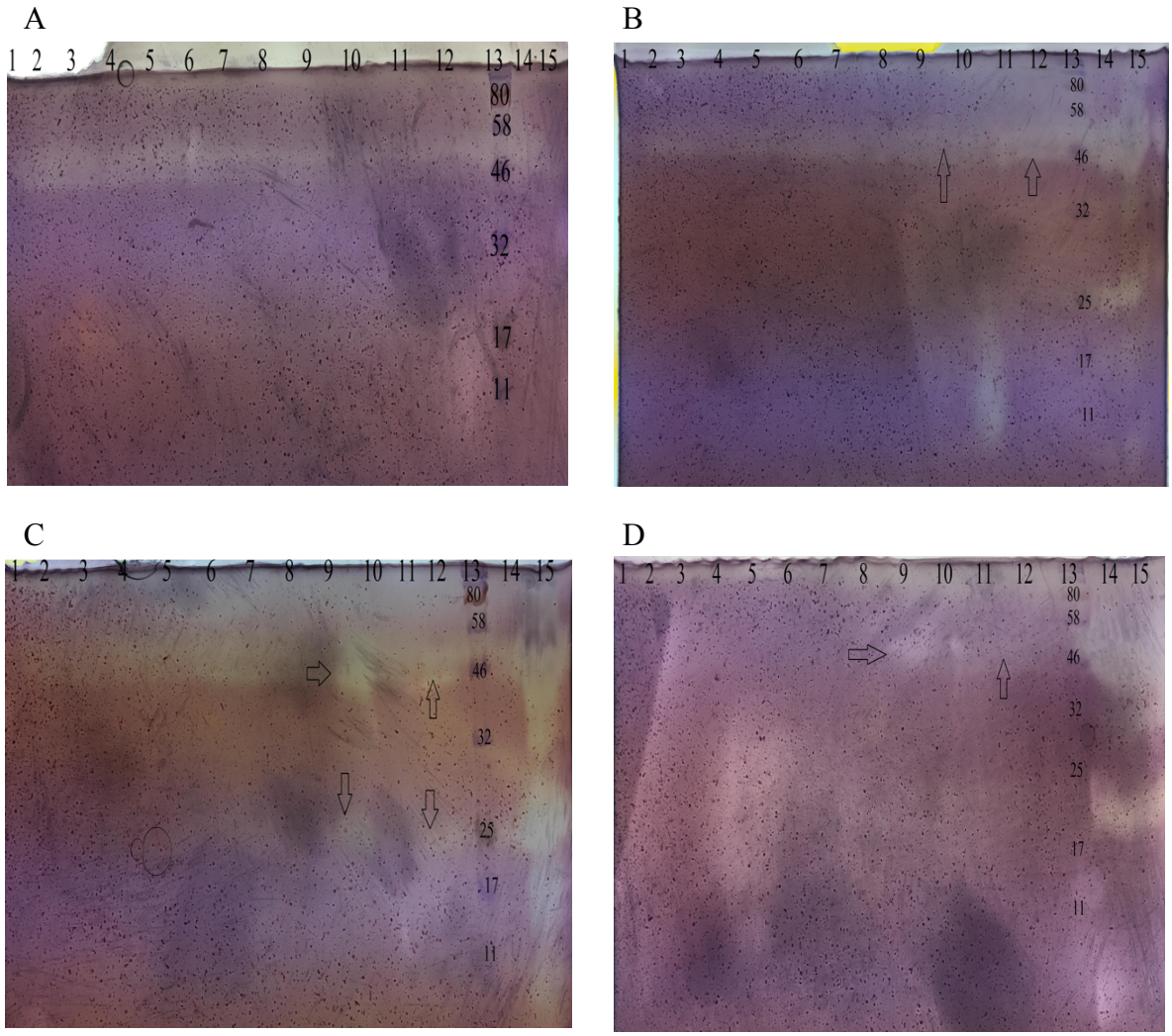


Figure 19. SDS-PAGE zymography of chromatographic fractions using MMC resin. (A) Zymography of 5X dil., 34.1 mS cm^{-1}, lane 1 - 7, flow through, lane 8 - 12 elution fractions. (B) Zymography of 9X dil., 18.7 mS cm^{-1}, lane 1 - 6 flow through, lane 7 - 12 elution fractions. (C) Zymography of 17X dil., 10.1 mS cm^{-1}, lane 1 - 7 flow through and lane; 8 - 12 elution fractions. (D) Zymography of 33X dil., 5.1 mS cm^{-1}, lane 1 - 6 as flow

through and lane 7 - 12 elution fractions. Lane 14, blank; lane 13 ladder and lane 15 was the reference enzyme (*T. reesie*, 50X), in all zymograms.

All of the sample loads, 5X, 9X, 17X and 33X dilution of CASP expressed similar chromatograms, but slightly larger elution peak was observed in the 17X and 33X diluted samples. The CMCase assay of the chromatograms fractions showed that the flow through and elution fractions were active towards CMC substrate (Figure 18, green bars). The elution fractions represented slightly higher activity than flow through. The higher CMCase activity was found in the elution fractions T14-T19 in all chromatographic runs of 3X, 5X, 17X and 33X diluted samples; approximately 0.030-0.036 U ml^{-1} and corresponding with the eluted protein peaks (UV 280).

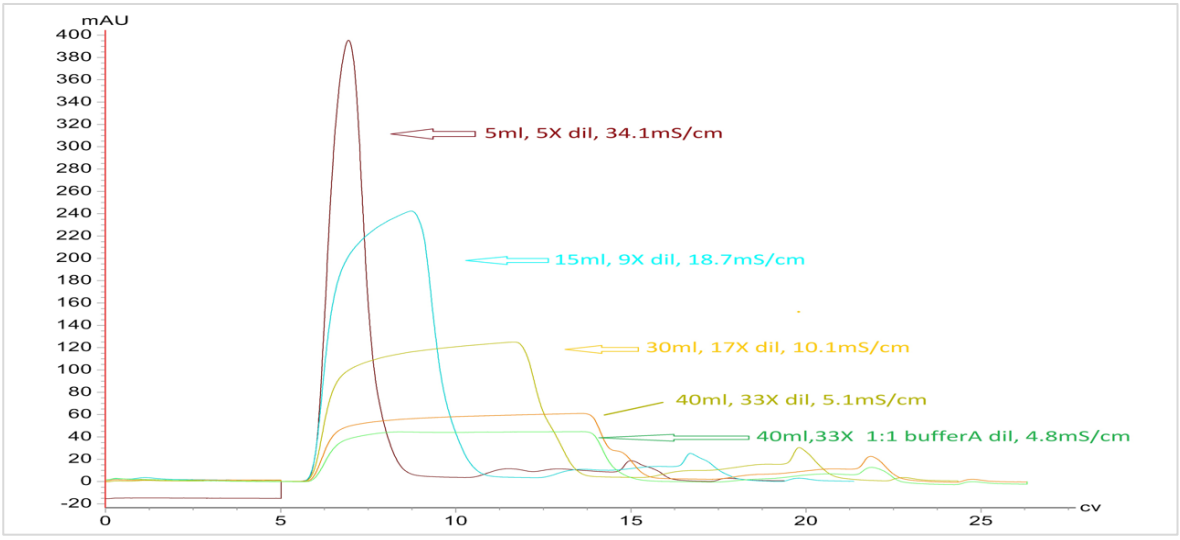
The zymography analysis of the flow through and eluted fractions after chromatography revealed that CMCase activity was present in most of the flow through and in the eluted fractions, except wash out fractions as shown in figure 19. In the zymography of 5X diluted CASP (figure 19 A), all flow through and elution fractions exhibited enzyme activity as halo bands at this position. The most prominent halo bands, however, were found in the flow through rather than in the elution fractions. For rest of the zymograms (figure 19 B, C and D) stronger bands were in the elution fractions indicating that more of the CMCase activity had adsorbed to the MMC column. Correspondingly, activity bands of flow through of the same chromatograms were very weak or nearly absent. Minor halo bands were observed at around 20 kDa to 25 kDa, but only in the zymography of 17X diluted CASP (lane 9 and 11, figure 19 C). These bands were absent in the rest of the zymography analysis.

3.11 Protein binding as function of conductivity

A comparison was made of the UV trace of five chromatograms from MMC column (Figure 20). The UV profile of the serially diluted CASP showed flow through phase increased in length with the larger and more diluted sample volumes, while the profiles during elution appear mostly similar. The relative amount of captured protein from the five different samples ware estimated in the Unicorn software by integrating the area (mAU * ml) below the UV profile to zero baseline, assuming same amount of sample was loaded in each run. The UV curves of the elution peaks were below 20 mAU in sample 5X, 9X, 33X diluted samples and in the buffer A 33X diluted sample. In the 17X diluted sample the elution peak was just higher than the 20 mAU, as shown in the figure 20 A.

The integrated areas below the elution profiles were then plotted against the sample conductivity (figure 20 B). The largest peak areas calculated with the zero-baseline integration was observed for the 17X diluted sample; 386 ml * mAU (appendix VII). The least peak areas were with the 33X buffer A and 33X distilled water diluted samples; 126 ml * mAU and 248 ml * mAU, respectively.

A



B

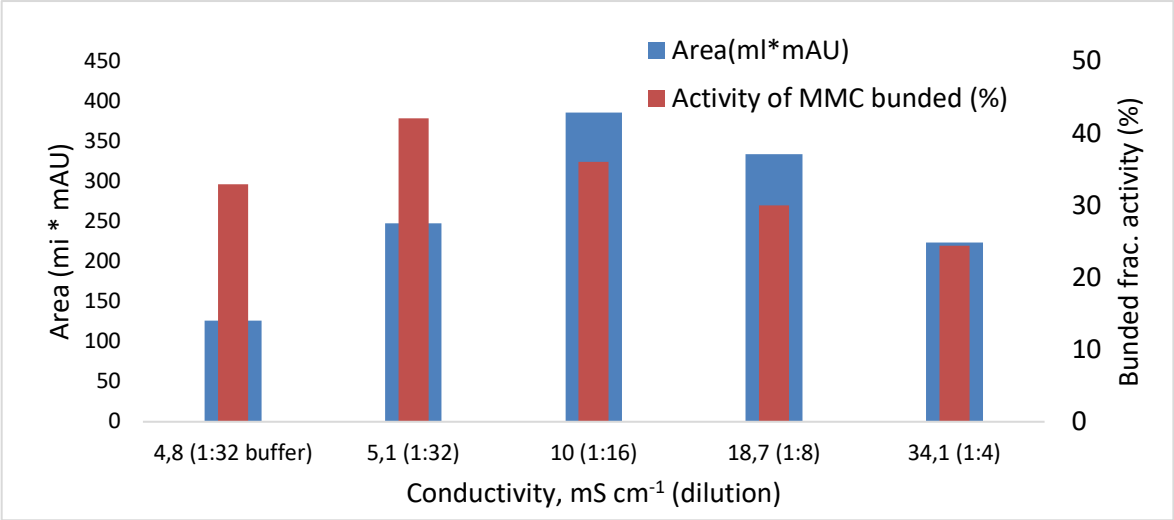


Figure 20. The effects of sample conductivity and eluted protein from the chromatography run on HiTrap Capto MMC column. (A) Comparison of UV profiles of chromatography performed under pH 4.7 and standard elution conditions, but different sample conductivity as well as sample loading volume. (B) Optimum sample binding conditions of captured protein (left axis) calculated by UV profile integration (mAU * ml). Compared is the corresponding to total CMCase activity of the eluted fractions (right axis).

This may be due to denaturation of sample proteins as diluted in the primary buffer with pH 4.3 or may be due to less ionic strength of distilled water diluted samples.

4. DISCUSSION

In nature, the degradation of cellulosic biomass is performed by mixture of hydrolytic enzymes collectively known as cellulases that are secreted by many microorganisms including fungi and bacteria (Dashtban et al., 2009). The cellulase enzymes are categorized as endo-acting (endogluconase), and exo-acting (cellobiohydrolase) that act synergistically for the degradation of cellulosic biomass. During synergistic reaction, cellobiose and cellodextrin - products of exo-gluconase and cellobiohydrolase - play inhibitory role towards the cellulose breakdown. Thus, the complete hydrolysis of cellulsoe via cellobiose to glucose requires beta-glucosidase that cleaves the final glycosidic linkage of cellobiose (Dashtban, Maki, Leung, Mao, & Qin, 2010).

The overall taxonomic composition of the moose rumen microbiome assessed by rRNA gene-encoding reads reveal that around 96.05 % were bacterial strains, 0.5 % archaeal and 3.62 % eukaryotic strains (Svartström et al., 2017). Out of these bacterial strains, *Bacillus species* were crucial cellulase secreting microorganisms and their expression depend upon the type of cellulosic substrate used in the culture media. The CMC and cellobiose are considered as the suitable substrate associated with cellulase production (Lambertz et al., 2014).

The media used for the cultivation of bacterial strain and the type of inducer selection for the secretion of extracellular enzyme determined the bacterial enzyme secretion. The media composition (carbon source, nitrogen source), presence of electrolyte and other essential factors as well as with external factors such as temperature, agitation, aeration and pH play key role for the optimal growth and maximum enzyme secretion. The secreted CMC acting enzyme proteins were analyzed by using verities of techniques such as DNS assay (reducing sugar) and SDS-PAGE zymography. The enzyme proteins were partly purified and captured by mixed mode chromatography.

4.1 Growth curve and CMCase activity

The finding and establishment of optimal bacterial growth conditions and maximum enzyme secretion is itself time consuming as well as difficult task. In the present experiment, the set up to find out the optimal bacterial growth with maximum enzyme activity using different sorts of media and various inducers as supplement.

Agitation vs. stationary culture: The experiments of MRB4 growth in ABB media with continuous agitation showed the positive effect to the cell density and CMCase production because it promotes the growth of microorganisms by providing homogenous distribution of nutrients, sufficient supply of oxygen and decrease in cell clumping. Comparatively, the bacterial cell density during shaking was higher than the culture performed without shaking. The cell density of the *MRB4* was started to increase after zero hour (t=0) and the highest peak obtained at 9 hours (figure 8 and figure 10) performed under shaking, and it remained up to 12 hours, but in stationary culture the MRB4 growth started after 2 to 3 hours. Similar to this study, the logarithmic phase in the Clare and co-workers (2017) started from zero hour and extended to the twelve hours, but it is noticed that the logarithmic phase in the current experiment was started faster than the previously studied *Bacillus sp*. by (Peixoto, Cladera-Olivera, Daroit, & Brandelli, 2011; Seo et al., 2013). Seo and co-workers (2013) study where the log phage of *B. licheniformis* was started from zero hours and the maximum cell density was obtained after 16 hours. The stationary phase began at hours of twelve which was similar to another *Bacillus sp.* studied by Clare and co-workers (2017) and the maximum cell density was obtained at 24 hours.

Rasp vs. PJ: The rasp as supplements during growth in ABB media showed positive impact, but was insufficient for growth and enzyme secretion because it only assists to increase bacterial optical density and enzyme secretion in a very low level, so it was excluded in further experimental work (Figure 9). The MRB4 growth in PJ instead of ABB media with rasp and glucose as supplements did not show the optimal bacterial growth (Figure 9) which is may be due to the lack of a nitrogen source, essential electrolytes and cofactors. The positive, but very low cell density was obtained at 30 ºC and at 40 ºC in PJ with rasp so the experiment with these ingredients was not proceed furthermore. Experiments performed by Kezeem and co-workers (2017) with various of agro-waste cocktails and Yang and co-workers (2014) with wet bran, corn powder, rice hull etc., as supplement for bacterial growth and enzyme production gave positive and significant effects on cell density as well as on CMCase secretion.

Growth curve on-set and length: The one-set of growth curve is totally depending upon the types of growth organisms and used media including other growth conditions. The bacterial growth started early when it cultured in enriched media at suitable temperature and continuous agitation.

Diauxic growth: Unlike the screening experiments performed in 250 ml shake baffled flask (figure 8), the large-scale fermentation experiments in the figure 11 shows the different growth curves under same conditions. The exhibited fermentation growth curve resembled diauxic type substrate consumption because the growth curve was completed in two steps during entire incubation period. The initial step was seen at around three hours of growth, then after addition of 0.5 % cellobiose, the cell density was declined gradually till seven hours of incubation then again, the cell density was rose again. The experiment performed by van Dyk and co-workers (2009) also reported the diauxic bacterial growth curve of *B. licheniformis* SVD1 during the entire incubation period. The growth curve obtained on this experiment (figure 11) was very unusual since these sorts of growth curves are usually obtain in the media containing two carbon energy sources.

CMCase and its rise and decline: When compared to the results of MRB4 cultivation performed in small scale and bench scale, differences were found in both 24 hours growth curves and CMCase activity as well. The CMCase activity was declined after 24 hours sample in small scale MRB4, while it was just opposite, and slightly increased in the large-scale fermentation I. In DoE based fermentation (F II) the CMCase activity was also declined after 24 hours sample. The studies performed by van Dyk and co-workers (2009) and Saratale and co-worker (2011) reported results similar to this observation where the CMCase activity was declined during stationary and death phase. The decrease in CMCase activity at that phase may be due to the metabolic repression caused by the molecules generated after hydrolysis of substrate by enzymes such as CMCase e.g. cellobiose, glucose etc. and by the proteolytic enzyme secretion. The CMCase activity generally found higher under shake condition because it favors bacterial growth than stationary growth conditions (figure 8 and figure 10). From this observation continuous agitation was considered to in cell density and CMCase activity. However, the on-set and length of the logarithmic phage in the MRB4 growth vary in condition like with shaking and without shaking, with and without supplements, but in experiment, the maximum cell density and CMCase activity ware found at 9 hours (sample harvesting time) of culture and extended upto12 hours then started another phase (Figure 8 and Figure 10). Similar to this study, *B. licheniformis* 2D55 in Kezeem and co-workers (2017) study also reached logarithmic phase at 6 to 12 hours with maximum CMCase activity. Then, stationary phase was remained for a few hours before starting to decline.

4.2 The comparison of effect of various supplements during fermentations

However, the cell density curves and the CMCase activity curves has similar pattern in both fermentation (figure 17) carried out in two different experiment under identical conditions except variation of the supplements in F II, but the individual values of cell density and CMCase activity during incubation was varied with each other. On the basis of effects of cellobiose in the F I, the DoE was set up and F II was carried out. The interesting evidence was that, the result of both fermentations ware similar i.e. increased in OD_{600} titer but decreased in CMCase titer at 24 hours. Similar kinds of results, the decrease of the CMCase activity in the final sample was also reported in the experiment performed by van Dyk and co-workers (2009), Seo and co-workers (2013) and Yang and co-workers (2014) and they concluded that the decrease in CMCase activity after prolonged cultivation may be due to the consumption and depletion of nutritional ingredient in the media and metabolite repression by molecule released after hydrolysis such as glucose or cellobiose and also with proteolytic effects until end of experiment. The drastic increased in CMCase titer immediately after addition of cellobiose could indicate the inducible nature of endoglucanase and induced in presence of cellulose derivative substrate like cellobiose to degrade it.

4.1.1 Effect of cellobiose on CMCase secretion

The medium for cellulase production generally contains variety of carbon sources. The cellulose derivative substrate like cellobiose showed positive effect on endoglucanase production i.e. CMCase. When 0.5 % w/v and 0.25 % w/v cellobiose was supplemented in fermentation I and II, respectively it caused significant increase in the CMCase activity. Then, it was considered that the CMCase activity could be due to the hydrolysis of cellobiose. Similar to this study Lynd and co-workers (2002) demonstrated the positive effect of cellobiose on CMCase secretion.

The fermentation based on the DoE screening revealed that the CMC substrate have a the significant as well as positive effect in the cell density, but not in the CMCase activity. Among the three different variables (figure 14), the CMC showed positive and significant effect ($p<0.001$ at 6 hrs. and at 24 hrs.) in cell density, but not in CMCase activity at 6 hours and 24 hours. However, at 6 hours, the $CaCl_2$ and temperature induced CMCase activity, but it was negative in both cell density and CMCase activity at 6 hours and 24 hours. The result reported

by the Thakkar and co-workers (2014) was contradictory to the effects observed in this experiment (figure 14) because they noted that CMC was the best endoglucanase inducer.

The better understanding of interaction between the variables and their response effects is on the optimal cell density and CMCase activity is shown in contour plots. The contour plots (figure 15) revealed that CMC play crucial role for the increased in the cell density. It showed the optimal cell density with higher CMC concentration and decreased in $CaCl_2$ to 10 mg L^{-1} in early incubation (t = 6) but declined in CMCase activity with increased in CMC percentage. While CMC had have positive effect on CMCase activity in Shajahn and co-workers (2017) study, it was negative and insignificant effect ($p<0.626$) at 24 hrs. and positive, but significant ($p<0.026$) at 6 hrs. effect in this study. The contour plot and the effect plots in this experiment revealed that the optimal CMCase activity was obtained when CMC concentration less than 2 % w/v i.e. 1 to 1.5 % w/v.

The difference in initial (t = 0) CMCase activity in F I and F II in the (figure 17), was due to the presence of 2 % w/v CMC in the F II as it gets different viscosity. The optical density that represent the cell growth was diauxic growth curve in both F I and F II experiments. After addition of cellobiose, the cell density was declined gradually till two to three hours then further incline in the cell density forming secondary peak. Similar to this study, van Dyk and co-workers (2009) reported the diauxic growth curve which may be due to the presence of secondary carbon source (cellobiose) during incubation period.

4.1.2 Effect of $Cacl_2$, and temperature in prolonged incubation

Similar to this experiment, the other experiments performed by Shahriarinour and co-workers (2011), reported that increased in cell density and the endoglucanase production was obtained on continuous shaking (Figure 8). The reason behind this reported by Shahriarinour and co-workers (2011) was that the continuous shaking assist to dissolve oxygen in the media, maintain homogeneous supply of nutrient and prevent clumping of cells. These are essential for the increase in cell density and enzyme secretion. However, the CMC substrate showed positive and significant effect in bacterial cell density with agitation, but not in the endoglucanase activity.

In the DoE screening the optimal bacterial cell density and the CMCase activity was better at 30 °C than at 40 °C, therefore the former was considered as the optimal temperature. However, the optimal temperature for better cell growth and enzyme secretion was varies according to the organism types and its habitat. For instance, thermophilic *Geobacillus sp.* produce thermostable endoglucanase that had maximum activity at 70 °C (Park et al., 2017). Unlike form this study, Acharya and co-workers (2012) reported that the best temperature for optimal cellulase production from *B. licheniformis MVS1* and *Bacillus sp.* MVS3 from hot spring was 50 °C and they also concluded that cellulase production declined significantly when temperature raised up above 60 °C. On the other hand, Seo and co-workers (2013) reported that the optimum temperature for *B. licheniformis JK7* endoglucanase activity found at 70 °C which was 0.75 U ml^{-1}. Besides that, they also reported *B. licheniformis JK7* expressed maximum glucosidase and xylanase activity at 50 °C, but not CMCase activity.

In this experiment, the various additives namely $CaCl_2$, Tween-20 and temperature at 30 ºC show the fluctuating effects on CMCase secretion and cell density. At 6 hours of incubation, the $CaCl_2$ compound expressed stimulatory effect on CMCase production, but the effect at 24 hours of incubation was just opposite. On the other hand, $CaCl_2$ did not show any effects on cell density like CMC substrate did on CMCase activity (Figure 15 and Figure 16). Although, $CaCl_2$ is a common additive used for optimization of bacterial cell density and CMCase activity, but here it showed the opposing effects. For instance, Seo and co-workers (2013) reported that $CaCl_2$ had inhibitory effect in CMCase activity, while Shahriarinour and co-workers (2011) reported stimulatory effect in CMCase activity.

The surfactant (Tween-20) included in the DoE and fermentation II expressed similar stimulatory effect on CMCase activity and cell density like it did in the experiment carried out by Seo and co-worker (2013) and Shahriarinour and co-workers (2011) and they clarified that the effect is due to increase in oxygen supply (lower surface tension and viscous media) and conformational changes in the enzyme structure. According to (Shahriarinour et al., 2011) experiments, the Tween-20 showed the stimulatory effect in cell density and CMCase activity, but other surfactant like Tween-80 and Triton X-100 play inhibitory role instead of stimulatory role in enzyme secretion and optical density. For example, 25 mg L^{-1} $CaCl_2$ showed less effect in CMCase activity than the 15 mg L^{-1} $CaCl_2$. Factors, $CaCl_2$, temperature and Tween-20 showed a stimulatory effect towards the CMCase secretion and cell density at 6 hours of incubation, but the effect was changed as the inhibitory at 24 hours sample. Over all, the effects

of various additives in this experiment was significant and transparent because the level of CMCase activity and cell density obtained at different time period of incubation in fermentation II was clearly higher than the fermentation I (Figure 17, Table 2 and appendix VIII).

4.2 Mixed mode chromatography for capture of CASP (endoglucanase) and CMCase

In order to isolate the CMCase active protein, the mixed mode chromatography (MMC) was employed. MMC is a crucial adsorption technique where the ligand displays more than one interaction mode such as ion-exchange, hydrophobic and hydrophilic bonding in the chromatographic processes. The target of the MMC in this experiment was to capture the CASP and search the answer of question; is it possible to isolate CMCase from other CASP of the fermentation (FI) sample?

The two chromatography buffers having different pH and salt, play essential roles for the binding and separation of the desired proteins. The pH of the mobile phase affects the charge states of both the protein and to a lesser degree on the ligand (Koningsveld, 2001). In this study, the pH of buffer A was maintained 4.5 and the pH of elution buffer was 7.5.

Because of the higher salt concentration or conductivity of the CASP solution and the negative effect it may have on the adsorption of protein on the resin, it was serially diluted for improving adsorption and to obtain larger elution peaks. There were not effective changes in the protein elution though serial decreases in conductivity. All the elution peaks were more or less similar, however, the loading sample with 10.1 mS cm^{-1} conductivity gave slightly larger elution peaks. than other peaks. The experiments performed by (Faruque, 2016) also did not find significant difference between the height of the eluted peak obtained at different conductivity, but at same pH. The chromatograms (figure 18) and the zymograms (figure 19) of different fractions (flow through, wash out unbound, and elution) confirmed the presence of CMCase in the elution fraction. In this experiment, the highest elution peak was obtained in the 17X (10.1 mS cm^{-1}) sample, but maximum CMCase activity was found in 34.1 mS cm^{-1}. On the other hand, the clear halo bands (50-55 kDa and 22-25 kDa) which represent the CMCase activity in this experiment was observed in the 10.1 mS cm^{-1} sample (Figure 19 and Figure 20). From the above result, the suitable conductivity was required for the maximum adsorption and elution of the CMCase from the mixed mode cation resin.

The zymogram (figure 12) of the FI crude sample and elution fraction of chromatography (figure 19) revealed the halo bands exactly in around the same size (50 kDa to 60 kDa) and other bands also obtained at around 20 kDa to 25 kDa molecular weight. This indicated that the cellulase in the crude concentrated sample in F I and MMC captured proteins tentatively belongs to same categories.

4.3 Suggestion for further work

A lot of work and study was performed on the MRB4 isolates and considerable information was gathered by the experimental work with its analysis, as well as supporting information from literature. In the future work on the MRB4 isolates the following work could be essential for optimal production, identification, purification and characterization of cellulase enzymes from moose rumen bacteria.

- Conforming identity of MRB4 isolate whether as *Bacillus* or *Coccobacillus* by repeating different biochemical test and genomic analysis (16S rDNA).
- Cultivation of MRB4 in high-cell density fermentation technique such as feed-batch cultivation for better yield of enzyme.
- Conforming of suitable pH, temperature, buffer and type of inducer for the maximum cell density and higher enzyme activity of CMCase and xylanase etc.
- Observation and selection of the inhibitory and stimulatory effects of different co-factors such as; CMC, cellobiose, other agro- waste using RSM to find out the optimal bacterial growth and enzyme yield.
- A broad range of MiMo resins are available on the market today, which are more expensive and intensive for protein separation.

5. CONCLUSION

In conclusion, the bacterial strain isolated from moose (*Alces alces*) rumen was designated as MRB4 was previously characterized as a facultative anaerobe *Bacillus* (Sanusi, 2013) and later as a *Coccobacillus* i.e. *Streptococcus sp*. (Rabiul, 2016). The latter genus was recently conformed from a parallel study at the University of Bergen (KO Strætkvern, pers. Comm.). After cultivation of MRB4 in different standard growth media (ABB, LB), an industrial waste fraction (potato juice) and with different supplements, it was discovered that MRB4 as mesophilic that favours starring and surfactant (Tween-20) for optimal bacterial growth and expression of CMCase activity. However, MRB4 showed variable response to the different response factors and supplements tested. The highest enzyme activity was reported after 9 hours to 12 hours of cultivation. From the result obtained by cultivation of MRB4 in small screening scale and with Design of Experiment to its bench scale fermentation on different media substrates, it appears that enzyme expression in MRB4 is inducible by cellobiose, while cell density by CMC and it can be predicted the isolate is able to regulate the expression based on the substrate used for its culture. In a previous study (Sanusi, 2013), it was demonstrated that it primarily expressed hemi-cellulolytic enzymes, but it was also confirmed that it can express endoglucanase activity predominantly.

After measurement of CMCase activity (DNS assay) and the zymography (CMC) of the crude, processed and purified supernatant of the MRB4 culture, it could be concluded that MRB4 is a cellulolytic bacteria that can induce enzyme for degradation of cellulosic substrate. This experimental work and the work and study from senior colleagues (Sanusi, 2013 and Rabiul, 2016) and also from various literature of (van Dyk et al., 2009, 2010b) demonstrated that MRB4 has cellulolytic properties. MRB4 displayed strong endoglucanase activity with distinct bands on zymograms at approximately in between 50 kDa to 60 kDa also at 20-25 kDa.

The initial purification of the endoglucanase from MRB4 isolate was performed using ammonium sulfate (AS) precipitation followed by capture on a MMC resin. The CMCase activity and the zymograpms of eluted fractions using salt gradient and pH shift demonstrated the capture and detection of endoglucanase in the AS-processed culture supernatant. The degree of protein and enzyme capture appeared to be a function of the sample conductivity in the range 5-31.1 mS cm^{-1}. The highest yield was at moderate salt (10 mS cm^{-1}) which is according to protein interaction predicated with mixed mode ligands. The molecular mass of

the captured endoglucanase in zymogram gave halo bands at around 50-55 kDa. The halo band also appeared at molecular mass of 20-25 kDa and is suspected to be xylanase.

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7. APPENDIX

List of Appendix

Appendix I Ingredients of ABB media and LB media

ABB media		LB media	
Compounds	gram $Liter^{-1}$	Compounds	Amounts
Peptone	16.0	Water	995 ml
Yeast extract	7.0	Tryptic soy broth	10.0 grams
Sodium chloride	5.0	NaCl	10.0 grams
Starch	1.0	Yeast extract	5.0 grams
Dextrose	1.0		
Arginine	1.0		
Sodium succinate	0.5		
L-cysteine HCl	0.5		
Sodium bicarbonate	0.4		
Ferric pyrophosphate	0.5		
Haemin	0.005		

Vitamin K	0.0005		
Sodium thioglycollate	0.5		
Sodium pyruvate	1.0		

II. Ingredients of DNS reagents

Compounds	Quantity
3, 5- dinitrosalicylic acid	10.6 g
Sodium hydroxide (NaOH)	19.8 g
Na-K tartarate	306 g
Phenol	7.6 ml
Na metabisulfite	8.3 g
Distilled water	1416 ml

III. Ingredients of 10% polyacrylamide gel.

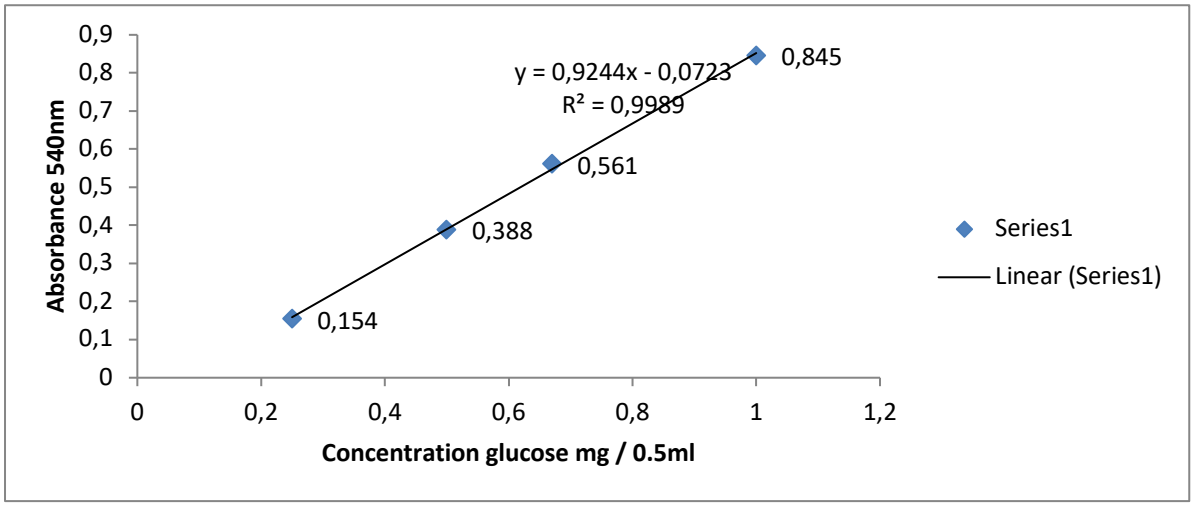
Solution	Protein stain gel	Zymography	Stacking gel (3 %)
40 % acrylamide stock	3 ml	3 ml	660 µl
Lower gel buffer	3 ml	3 ml	-
Upper gel buffer	-	-	1.66 ml
RO water	6 ml	4.8 ml	4.2 ml
1 % substrate solution (CMC)	-	1.2 µl	-
10 % ammonium persulfate (fresh)	50 µl	50 µl	100 µl
TEMED	8 µl	8 µl	5 µl

Note: The protein gel and the zymography gel was prepared in half the quantity of the ingredients in single test tube.

IV. Glucose calibration curve.

Glucose concentration (U ml^{-1})	Absorbance (540 nm)
0,25	0,154
0,5	0,388

0,67	0,561
1	0,845



V. The chromatographic separation of 24 hours processed MRB4 fermentation samples using MMC resin.

1. Mixed mode chromatography (Capto MMC^{TM} ,5 ml, 5X dil, 34.1 mS cm^{-1})

M	C	Fractions no.	CMCase activity (U ml^{-1})	Glucose (mg ml^{-1})
0,9244	0,0723	T2	0,029	0,079294678
0,9244	0,0723	T4	0,031	0,084703592
0,9244	0,0723	T5	0,030	0,081458243
0,9244	0,0723	T8	0,025	0,069558633
0,9244	0,0723	T9	0,029	0,079294678
0,9244	0,0723	T11	0,030	0,082540026
0,9244	0,0723	T14	0,031	0,085785374
0,9244	0,0723	T15	0,034	0,093357854
0,9244	0,0723	T16	0,034	0,092276071
0,9244	0,0723	T17	0,033	0,090112505
0,9244	0,0723	T18	0,032	0,086867157

2. Mixed mode chromatography (Capto MMC^{TM} ,15 ml, 9X dil, 18.7 mS cm^{-1})

M	C	Fractions no.	CMCase activity (U ml^{-1})	Glucose (mg ml^{-1})
0,9244	0,0723	T2	0,029	0,08037646

0,9244	0,0723	T3	0,029	0,079294678
0,9244	0,0723	T5	0.028	0,078212895
0,9244	0,0723	T6	0,030	0,082540026
0,9244	0,0723	T8	0,029	0,079294678
0,9244	0,0723	T9	0,030	0,081458243
0,9244	0,0723	T13	0,030	0,083621809
0,9244	0,0723	T14	0,029	0,08037646
0,9244	0,0723	T15	0,031	0,084703592
0,9244	0,0723	T16	0,034	0,093357854
0,9244	0,0723	T17	0,035	0,095521419
0,9244	0,0723	T18	0,032	0,08794894
0,9244	0,0723	T19	0,033	0,090112505

3. Mixed mode chromatography (Capto MMC^{TM} ,30 ml, 17X dil, 10.1 mS cm^{-1})

M	C	Fractions no.	CMCase activity (U ml^{-1})	Glucose (mg ml^{-1})
0,9244	0,0723	T3	0,030	0,083621809
0,9244	0,0723	T4	0,029	0,08037646
0,9244	0,0723	T7	0,030	0,081458243
0,9244	0,0723	T10	0,031	0,085785374
0,9244	0,0723	T11	0,031	0,084703592
0,9244	0,0723	T12	0,029	0,079294678
0,9244	0,0723	T14	0,031	0,084703592
0,9244	0,0723	T16	0,033	0,090112505
0,9244	0,0723	T17	0,034	0,094439637
0,9244	0,0723	T18	0,035	0,096603202
0,9244	0,0723	T19	0,033	0,091194288
0,9244	0,0723	T20	0,033	0,090112505
0,9244	0,0723	T21	0,034	0,093357854

4. Mixed mode chromatography (Capto MMC^{TM} ,40 ml, 33X dil, 5.1 mS cm^{-1})

M	C	Fractions no.	CMCase activity (U ml^{-1})	Glucose (mg ml^{-1})
0,9244	0,0723	T2	0,031	0,084703592
0,9244	0,0723	T3	0,029	0,079294678
0,9244	0,0723	T4	0,030	0,081458243
0,9244	0,0723	T5	0,030	0,082540026
0,9244	0,0723	T6	0,031	0,084703592
0,9244	0,0723	T9	0,032	0,086867157
0,9244	0,0723	T10	0,029	0,079294678
0,9244	0,0723	T11	0,030	0,081458243
0,9244	0,0723	T12	0,029	0,079294678
0,9244	0,0723	T13	0,032	0,086867157
0,9244	0,0723	T14	0,030	0,082540026
0,9244	0,0723	T15	0,033	0,090112505
0,9244	0,0723	T16	0,034	0,092276071
0,9244	0,0723	T17	0,032	0,08794894
0,9244	0,0723	T18	0,034	0,094439637
0,9244	0,0723	T19	0,035	0,096603202
0,9244	0,0723	T20	0,034	0,092276071
0,9244	0,0723	T21	0,032	0,086867157

VI. DoE for optimization of MRB4 growth and CMCase activity by RSM.

Experiment	**Media**	**CMC, 2 % w/v**	**Cacl2, 25 mg L^{-1}**	**Temperature (ºC)**	**CMCase, media as sample**	**OD600, 6 hours**	**Cmcase, 6 hours**	**OD600, 24 hours**	**CMCase, 24 hours**
ABB media									
1	R1	0	0	40	0,548	0,019	0,368	0,048	0,269
	R1´	0	0	40		0,012	0,401	0,074	0,342
2	R2	2	0	30	0,56	0,312	0,331	0,54	0,286
	R2´	2	0	30		0,32	0,32	0,545	0,228
3	R3	0	25	30	0,539	0,017	0,411	0,158	0,293
	R3´	0	25	30		0,02	0,382	0,162	0,21
4	R4	2	25	40	0,562	0,223	0,521	0,514	0,26
	R4´	2	25	40		0,256	0,441	0,484	0,258
LB media									
1	R1	0	0	40	0,212	0,013	0,189	0,03	0,2

	R1´	0	0	40		0,016	0,201	0,029	0,185
2	R2	2	0	30	0,206	0,016	0,191	0,118	0,041
	R2´	2	0	30		0,019	0,18	0,117	0,032
3	R3	0	25	30	0,211	0,031	0,229	0,128	0,027
	R3´	0	25	30		0,042	0,183	0,14	0,034
4	R4	2	25	40	0,21	0,052	0,194	0,164	0,036
	R4´	2	25	40		0,057	0,203	0,162	0,034

VII. Dilution and conductivity with measurement of total areas of peak (zero baseline) with CMCase activity of bonded fractions.

Dilution	Conductivity (mS cm^{-1}	Initial CMCase activity (U ml^{-1})	Laded volume (ml)	Total loaded activity (U)	Total activity of eluted (U) x 2	Activity of bunded enzyme (%)
5X	34.1	0.03	5 ml	0.15	0.614	24.4
9X	18.7	0.016	15 ml	0.24	0.798	30
17X	10.0	0.01	30 ml	0.30	0.626	36.31
33X	5.1	0.009	40 ml	0.37	1.134	42
33X A	4.8	0,012	40 ml	0.48	1.44	33.4

VIII. Cell density corresponding CMCase activity growth fermenter I and growth fermentor II.

Time (hour)	Growth fermentor I	CMCase activity I	Growth fermentor II	CMCase activity II
0	0,024	0,154554423	0	0,078
1	0,066	0,168593886	0,055	0,082
2	0,101	0,150944276	0,063	0,079
3	0,126	0,165384866	0,041	0,09
4	0,141	0,17260516	0,194	0,077
5	0,129	0,310191891	0,372	0,083
6,6	0,134	0,309790763	0,42	0,088
7	0,076	0,329044883	0,406	0,104

8	0,081	0,315406548	0,531	0,437
9,5	0,13	0,302169341	0,715	0,348
10	0,165	0,304174978	0,681	0,445
11	0,182	0,317011058	0,631	0,436
12			0,689	0,47
13			0,698	0,436
24	0,435	0,349	1,91	0,238

IX. ANOVA table obtained by using RSM at 6 hours sample on ABB media.

Optical density~	**DF**	**SS**	**MS (variance)**	**F**	**p**	**SD**
Total	7	27,9798	3,99712			
Constant	1	27,9798	27,9798			
Total corrected	6	2,33881e-006	3,89801e-007			0,000624341
Regression	3	2,32782e-006	7,75941e-007	211,914	**0,001**	0,000880875
Residual	3	1,09847e-008	3,66158e-009			6,0511e-005
Lack of Fit (Model error)	0	9,77884e-013	--	--	--	--
Pure error (Replicate error)	3	1,09838e-008	3,66125e-009			6,05083e-005
	N = 7	Q2 = --		Cond. no. = 1,414		
	DF = 3	R2 = --		RSD = 6,051e-005		
		R2 adj. = --				
CMCase~	**DF**	**SS**	**MS (variance)**	**F**	**p**	**SD**
Total	8	1,35182	0,168977			
Constant	1	1,31938	1,31938			
Total corrected	7	0,0324412	0,00463446			0,0680769
Regression	3	0,0285122	0,00950408	9,67584	**0,026**	0,0974889
Residual	4	0,00392899	0,000982248			0,0313408
Lack of Fit (Model error)	0	4,65661e-010	--	--	--	--
Pure error (Replicate error)	4	0,00392899	0,000982248			0,0313408

	N = 8	Q2 = --	Cond. no. = 1	
	DF = 4	R2 = --	RSD = 0,03134	
		R2 adj. = --		

X. ANOVA table of 24 hours sample performed on ABB media based on RSM

Optical density~	DF	SS	MS (variance)	F	p	SD
Total	8	57,2632	7,1579			
Constant	1	55,9987	55,9987			
Total corrected	7	1,2645	0,180643			0,425021
Regression	3	1,2464	0,415466	91,7995	**0,000**	0,644566
Residual	4	0,0181032	0,0045258			0,067274
Lack of Fit (Model error)	0	1,86265e-009	--	--	--	--
Pure error (Replicate error)	4	0,0181032	0,0045258			0,067274
	N = 8	Q2 = --	Cond. no. = 1			
	DF = 4	R2 = --	RSD = 0,06727			
		R2 adj. = --				
CMCase	**DF**	**SS**	**MS (variance)**	**F**	**p**	**SD**
Total	8	0,587218	0,0734023			
Constant	1	0,575665	0,575665			
Total corrected	7	0,0115535	0,0016505			0,0406264
Regression	3	0,0037605	0,0012535	0,643397	**0,626**	0,0354048
Residual	4	0,007793	0,00194825			0,044139
Lack of Fit (Model error)	0	0	--	--	--	--
Pure error (Replicate error)	4	0,007793	0,00194825			0,044139
	N = 8	Q2 = --	Cond. no. = 1			
	DF = 4	R2 = --	RSD = 0,04414			
		R2 adj. = --				