

**Isolation and characterization of novel cellulase and
pectinase producing bacteria**

A thesis presented to
The Faculty of Graduate Studies
of
Lakehead University
by

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In partial fulfillment of requirements for the degree of
Master of Science in Biology

July 20, 2015

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ABSTRACT

Cellulases and pectinases are the major cell wall degrading enzymes. The microorganisms producing these enzymes have a wide range of industrial applications. In this research, 17 bacterial isolates from rotting wood samples were screened for their cellulase activity by carboxymethyl cellulose (CMC) plate assay. Similarly, a bacterial strain isolated from the gut of western honey bee (*Apis mellifera* L.) showed polygalacturonase activity by pectin agar plate assay. The bacterial isolates showing higher region of depolymerisation were further assayed for their activities for producing the enzymes quantitatively and they were identified on the basis of 16S rDNA sequence analysis. The protein gel was run using SDS-PAGE for molecular weight determination of the cellulase and polygalacturonase. The sequences of two isolates producing cellulase (*Bacillus* sp K1 and *Bacillus* sp. A0) and one isolate producing polygalacturonase (*Bacillus* sp. HD2) were successfully uploaded to the NCBI data base. The enzymes produced by isolates K1 and HD2 were characterized. The isolate K1 produced the maximum CMCase at pH 6 and 50 °C in presence of peptone (1%) as a source of nitrogen. The enzyme activity was stimulated by Ca²⁺ (2 mM) by 20% over the control. *Agave* biomass was fermented by using two cellulase producing isolates K1 and A0 and ethanol was detected by using micro-dichromate method. Both the strains produced ethanol using untreated *Agave* biomass. Similarly, the polygalacturonase produced by HD2 strain exhibited enzyme activity in a wide range of pH from pH 5-12. The production was enhanced by using yeast extract (3%) in the production medium and the enzyme activity was stimulated by Ca²⁺ (2 mM) and SDS (200 mM). In SDS-PAGE gel, the molecular weights of cellulase enzymes produced by K1 and A0 were ~36 kDa and ~40 kDa respectively and the two clear bands of polygalacturonases produced by isolate HD2 were found at ~36 kDa and ~72 kDa.

LAY SUMMARY

Faculty and students in the Department of Biology are bound together by a common interest in explaining the diversity of life, the fit between form and function, and the distribution and abundance of organisms. The present research shows the isolation and characterization of industrially important cellulase and pectinase (polygalacturonase) producing bacterial strains. Two bacterial isolates (*Bacillus* sp K1 and *Bacillus* sp. A0) isolated from rotting wood samples and one bacterial isolate (*Bacillus* sp HD2) isolated the gut of western honey bee (*Apis mellifera* L.) efficiently produced cellulase and polygalacturonase respectively. This research characterized the working parameters of these enzymes showing their possible applications in different industries. Further, the production of bioethanol by using untreated *Agave* biomass demonstrated these cellulase producing *Bacillus* strains' ability for the efficient use in biofuel production.

ACKNOWLEDGEMENTS

I am very much grateful to my supervisor Dr. Wensheng Qin for his continuous support and encouragement. I would like to express my deep sense of gratitude, appreciation and indebtedness for the opportunities he has given to me during my study and research.

I am also grateful to my research committee members; Dr. Kam T. Leung and Dr. Robert Mackereth; and external reviewer Dr. Ze-Chun Yuan for the guidance, helpful suggestions and comments on my thesis. I thank Dr. Guosheng Wu for helping me to take SEM images. I also thank other professors and graduate students for their valuable suggestions and comments during my studies at Lakehead University.

I am thankful to Dr. Miranda Maki, Dr. Chaoyang Lin, Shreeti Pradhan, Md. Shafiqur Rahman and other lab mates for their help and suggestions on my research. Finally, I thank my family and friends for encouraging me to pursue this research.

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CHAPTER I

General introduction: cellulose and pectin degrading enzymes and their applications

Abstract

Cellulose and pectin are the major plant polysaccharides. These polysaccharides provide structural integrity to the plant cell. The enzymatic hydrolysis of plant polymers using cellulases and pectinases has received attention in the last decades because of their possible uses in different industries such as pulp and paper, food and feed, agriculture, bioconversion, textile and others. However, it is challenging to develop low cost effective enzymes for saccharification of the polysaccharides. Many microorganisms including fungi and bacteria are capable of producing the enzymes such as cellulases and pectinases for the degradation of cellulose and pectin respectively. So, it is very important to isolate and characterize the bacteria producing such industrially important enzymes as they offer several benefits over other microorganisms. Further, the induction of enzymes under different conditions will also be useful for the potential industrial applications of these enzymes.

Key words: Cellulase, pectinase, microorganisms, applications

1. Introduction

Plant cells contain a number of polysaccharides. The major polysaccharides include starch, cellulose, hemicelluloses and pectin. These polysaccharides provide structural integrity to the plant cell. Their degradation is important for different industries including textile, paper and pulp, food, feed, beverage, as well as several other industrial processes. The plant cell wall polysaccharides such as lignocellulose and its derivatives have been shown as a major feedstock for the sustainable production of environmentally friendly fuels and chemicals (Knauf and Moniruzzaman 2004). Moreover, the plant carbohydrate polymers can also be used as substrates for important enzymes such as cellulases and pectinases. Recently, enzymatic degradation of these polymers has received attention as they have been found more attractive alternative to chemical and mechanical processes. There has been some progress in identifying and characterizing the microorganisms which produce the enzymes for the degradation of plant polysaccharides. Mainly the fungal and bacterial strains are commonly used for the production of polysaccharide-degrading enzymes as these microorganisms encode a wide spectrum of cell wall-degrading enzymes (Juturu and Wu 2014). Due to this, there is an increasing demand for replacing the traditional physical and chemical processes with advanced biotechnological processes involving microorganisms and enzymes like cellulases and pectinases (Bajpai 1999).

1.1 Cellulose

The lignocellulosic materials in plants are mainly composed of cellulose, hemicellulose and lignin. Cellulose is the major constituent of plant cell wall. It is a water insoluble polysaccharide composed of repeated units of glucose (Fig. 1) which are linked with β -1, 4 glycosidic bonds (Heredia et al. 1995). The number of the glucose monomers in the cellulose molecules varies and the degree of polymerization ranges

from 250 to over 10,000 depending upon the source and method of treatment (Klemm et al. 2005). Cellulose is a crystalline polymer and the chains in the crystal are connected together by intra and inter chain hydrogen bonds. The adjacent sheets are held together by Van-der Waals force. In most of the cases in nature, cellulose fibers are present together with the matrix of other biopolymers, mainly hemicellulose and lignin (Lynd et al. 1999), where glucose units are embedded in a hemicellulose, pectin and lignin matrix. In polymeric structure of cellulose, it has crystalline and amorphous regions along with several surface irregularities (Cowling 1975; Fan et al. 1980). This feature makes the cellulosic fibers capable of swelling when hydrated partially forming the micro pores and cavities sufficiently large enough for the penetration of large molecules like enzymes.

The cellulosic fibers are most abundantly found in the biomass of plants. For the utilization of plant's cellulosic material, depolymerisation of cellulose to glucose units is a prerequisite. The cellulose is resistant to degradation due to its crystalline nature, however this can be achieved by physical, chemical or enzymatic hydrolysis. Physical and chemical hydrolysis requires expensive equipment and energy and has environmental concerns and nonselective by products formation (Palmqvist and Hahn-Hangerdal 2000). To overcome these hurdles, enzymatic hydrolysis is the best alternative for the hydrolysis of lignocellulosic biomass.

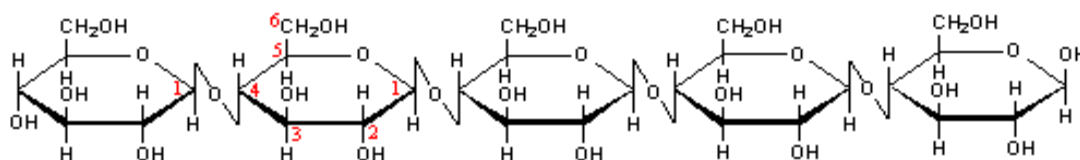


Figure 1 Schematic representation of cellulose

The main sources of cellulose with the potential application are the agricultural wastes which are the residues left after the processing, production and harvest of

cereals, fruits, vegetables and trees. Every year they are produced in a large amount throughout the world and generally either used for animal feed or burnt in the fields. These agricultural wastes are used as effective substrates for enzyme production by solid-state fermentation (Martin et al. 2004) and for the production of value added products such as organic acids, biofuels, protein rich feed, aroma compounds and bioactive secondary metabolites (Nigam and Pandey 2009). Due to the consumption of petroleum based fuels, plant lignocellulosic biomass is obtained as agricultural by products and industrial residues is the best alternative to produce renewable, abundant, environmentally friendly feedstock for the production of biofuels through biorefinery process (Menon and Rao 2012). However, it is very competitive and challenging to liberate the sugars from lignocellulosic biomass for the production of biofuels and value added bio-based chemicals because the lignocellulosic biomass is recalcitrant to microbial action. This problem can be solved by suitable pre-treatments by disrupting the lignin structure which increases the enzymes accessibility and enhance the rate of biodegradation (Lynd et al. 2002).

1.1.1 Cellulose degrading enzymes

Since cellulose is the major structural polysaccharide in plant cell and the most abundant organic material on the earth (Brown 2004), the enzymes degrading cellulose or the microorganisms releasing such enzymes play an important role in global carbon cycle. Microbial degradation of the cellulose in lignocellulosic biomass and organic waste is accompanied by an action of several enzymes, the most important of which are the cellulases. These enzymes help to breakdown the β -1, 4 glycosidic bonds in cellulose polysaccharide. Mechanistically, there are three types of cellulases which act synergistically for the complete hydrolysis of cellulose. These enzymes are endo-(1,4)- β -D-glucanase (EC 3.2.1.4), exo-(1,4)- β -D-glucanase (EC 3.2.1.91), and β -

glucosidases (EC 3.2.1.21) (Deswal et al. 2011; Kuhad et al. 1997). The endoglucanase randomly breaks the internal O-glycosidic bonds which results the formation of glucan chains of different lengths; the exo glucanase acts on the ends of the cellulose chains resulting the formation of β -cellobiose as end products and the β -glucosidase attacks the β -cellobiose or small polysaccharides (Fig. 2) to produce glucose molecules (Bayer et al. 1994; Perez et al. 2002). These cellulases have been used for both academic research and industrial production.

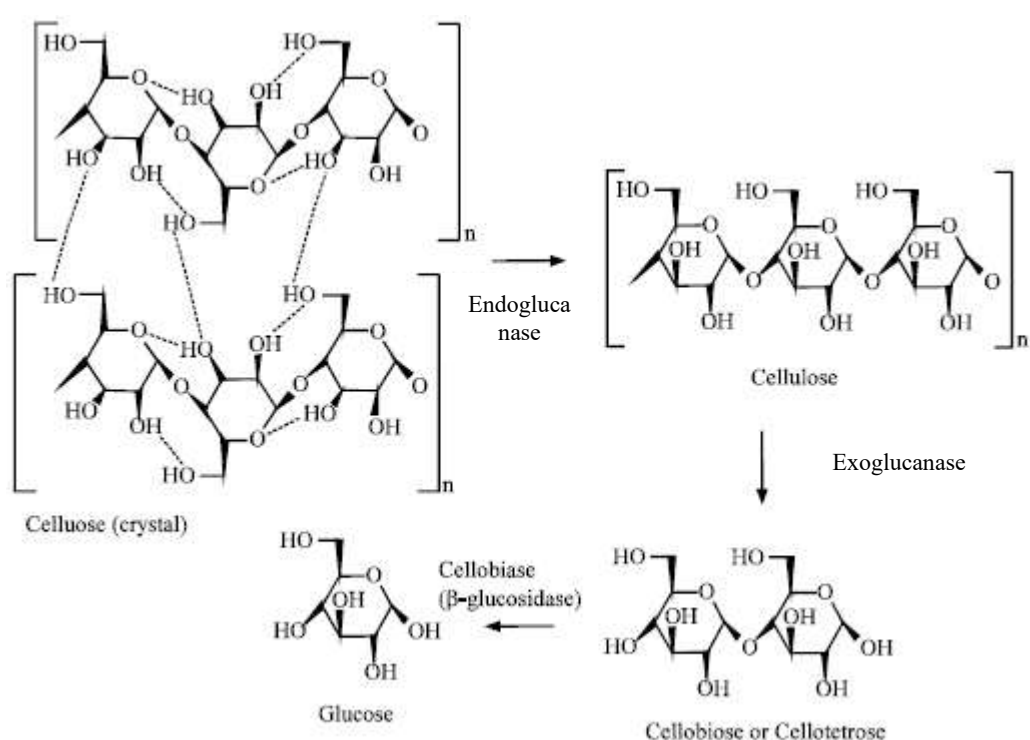


Figure 2 Cellulose degrading enzymes; cited from (Karmakar and Ray 2010)

1.2 Pectin

Pectin is a high molecular weight polysaccharide present as the major component of middle lamella and primary cell walls of higher plants. It is also present in the junction zone between cells with secondary walls. It provides firmness and structural integrity to the tissues (Ridley et al. 2001) and is composed of D-galacturonic acid units linked together by α -1-4 glycosidic bonds (Fig. 3). Some of these units are

modified by methyl esterification which occurs at the carboxyl groups at O-6 or acetyl esterification at hydroxyl groups at O-2 or O-3 position (de Vries and Visser 2001). In plant cell, pectin is embedded in cellulosic microfibrils and provides rigidity to cell walls. During fruit ripening, the pectin is structurally altered by naturally occurring pectinase enzymes which breakdown the chains of pectin; as a result of this pectin becomes more soluble and its cementing ability to the surrounding cell wall is loosened and the tissue becomes soft. The pectic substances account for 0.5-4.0% of the fresh weight of plant material (Sakai et al. 1993); and when the tissue is ground, the pectin becomes soluble which increases the viscosity and pulp particles where as other pectin molecules remain bounded with hemicellulose and facilitate water retention.

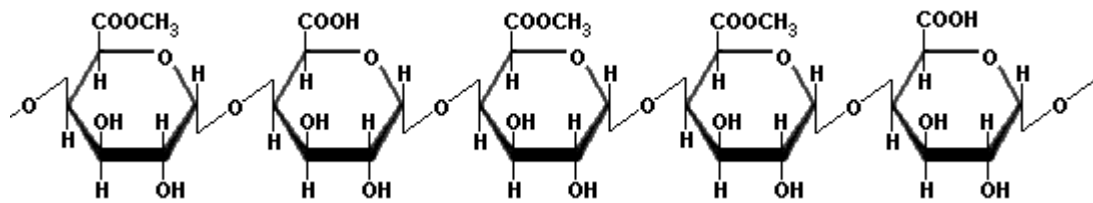


Figure 3 Schematic representation of pectin

In addition to its roles in plant growth, development and plant defense, pectin functions as a gelling and stabilizing agent in many food and cosmetic products and has many applications in pharmaceutical industries producing a variety of products including surface modifier for medical devices and materials for biomedical implantation (Mohnen 2008). Also, there has been tremendous progress in understanding of the pectin structure with the application of techniques such as enzymatic fingerprinting, mass spectrometry, NMR and molecular modelling which indicate the possible plant agronomical properties of this polymer (Mohnen 2008; Willats et al. 2006).

1.2.1 Pectin degrading enzymes

Pectin degrading enzymes are known as pectinases or pectinolytic enzymes. These enzymes are heterogeneous groups of related enzymes that hydrolyse pectin or pectic substances. They mainly occur in higher plants and microorganisms (Whitaker 1990). Microbial pectinases play important role in plant pathogenesis, symbiosis and breaking down of plant pectin for nutritional purposes of pathogens (Lang and Dornenburg 2000). They also help in cell wall extension and softening of plant tissues during maturation and storage (Sakai and Winkelmann 1992). Pectinases are produced by many microorganisms like bacteria, fungi and yeasts using submerged culture fermentation and solid-state fermentation.

The hydrolysis of pectin backbone is obtained by the synergistic action of several enzymes (Table 1). On the basis of their mode of action, pectinases are classified into three major groups: polygalacturonase (PG), pectin lyase (PL) and pectin esterase (PE) (Fig. 4). Polygalacturonase hydrolyses α -1, 4 glycosidic linkages by both exo and endo mechanisms and are the most abundant among all the pectinases. They are further classified into endo polygalacturonase (EC 3.2.1.15) and exo polygalacturonase (EC 3.2.1.67) which hydrolyse the internal and external (1, 4) glycosidic linkages of pectin respectively. Pectin lyase (EC 4.2.2.10) splits (1,4) glycosidic bonds by trans elimination, which results in galacturonate with double bond between C-4 and C-5 at the non-reducing end, while pectin esterase (EC 3.1.1.11) catalyses the hydrolysis of methyl group to produce pectin and methanol (Gummadi and Panda 2003; Sharma NR 2011).

Table 1. Enzymes for pectin degradation

Enzymes	EC Number	Mechanism
Pectin lyase	4.2.2.10	Random eliminative cleavage of (1-4)- α -D-galacturon methyl ester to give oligosaccharides with 4-deoxy-6-O-methyl- α -D-galact-4-enuronosyl groups at their non-reducing ends
Pectate lyase	4.2.2.2	Random eliminative cleavage of (1-4)- α -D-galacturon to give oligosaccharides with 4-deoxy- α -D-galact-4-enuronosyl groups at their non-reducing ends
Pectate disaccharide lyase	4.2.2.9	Eliminative cleavage of 4-(4-deoxy- α -D-galact-4-enuronosyl)-D-galacturonate from the reducing end of pectate (i.e. de-esterified pectin)
Pectate trisaccharide lyase	4.2.2.22	Eliminative cleavage of unsaturated trigalacturonate as the major product from the reducing polygalacturonic acid/pectate
Endo-polygalacturonases	3.2.1.15	Random hydrolysis of (1-4)- α -D-galacturonic linkages in pectate and other galacturonans
Exo-polygalacturonases	3.2.1.67	Hydrolysis of D-galacturonic acid residues from the reducing ends of polygalacturonate chains
Pectin methyl esterases	3.1.1.11	Demethoxylation of pectin, forming pectate
Pectin acetyl esterases	3.1.1.6	Deacetylation of pectin, forming pectate

Source- Modified from (Biz et al. 2014)

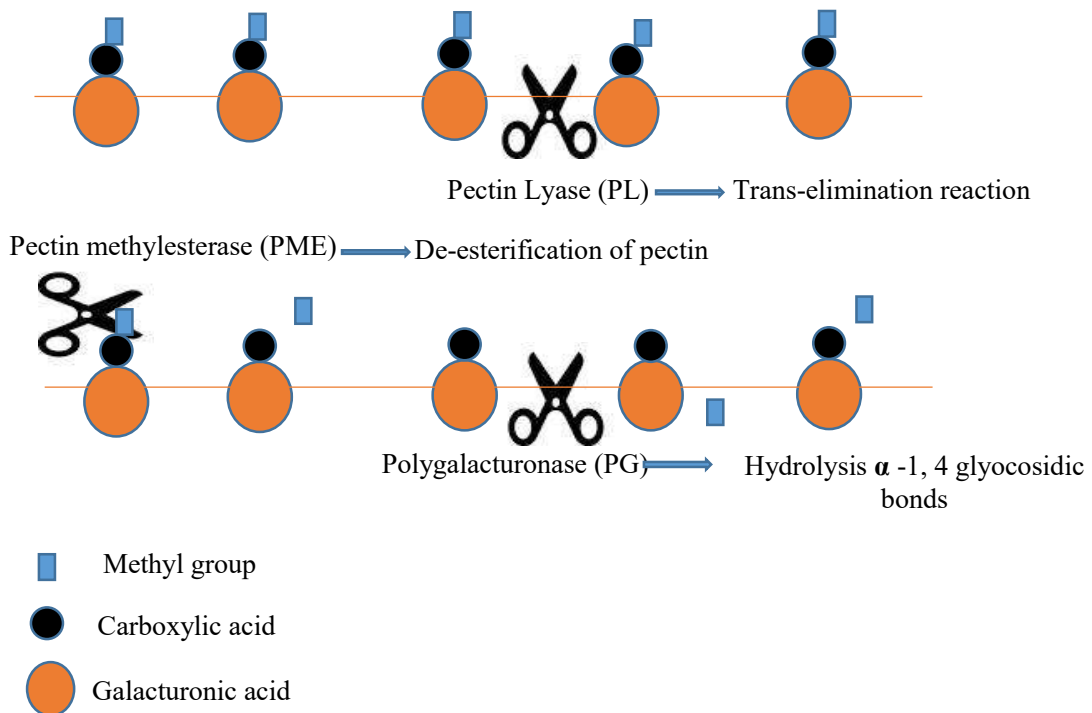


Figure 4 Mode of action of pectinases

The production of microbial pectinases became prominent for many decades. Among all the pectinases, polygalacturonases are widely used in different industries. At a commercial scale, usually fungal polygalacturonases have been used. The pectinases with novel properties from bacterial origin have advantages over the fungal pectinases because the enzyme production is achieved in less time due to fast bacterial growth compared to fungi.

1.3 Industrial applications of the cellulases and pectinases

Cellulases and pectinases have wide applications in different industries. These enzymes help to degrade the plant polysaccharides producing reducing sugars. Further, the reducing sugars are converted to different value added products. These enzymes also help in the processing different industrial products. Because of this feature, currently cellulases and pectinases have been used in pulp and paper industry, textile industry, washing powder industry, food and animal feed (Table 2).

Table 2. Industrial applications of cellulases and pectinases

Enzymes	Industry	Applications
Cellulases and Pectinases	Agriculture	Biocontrol of plant pathogens and diseases, production of plant and fungal protoplasts, enhancing seed germination and improvement of root system, enhancing plant growth and flowering, improvement of soil quality
Cellulases and Pectinases	Animal feed	Improvement of the nutritional quality of animal feed, improvement in feed digestion and absorption, production and preservation of high quality fodder
Cellulases	Bioconversion	Conversion of cellulosic materials to ethanol, other value added products, organic acids and lipids
Cellulases	Detergents	Cellulase based washing powders have superior cleaning action without damaging fibers with improved color brightness and dirt removal, removal of rough protuberances in cotton fabrics, bio stoning of denim fabrics, bio polishing of cotton and non-denim fabrics, production of high quality fabrics
Cellulases and Pectinases	Fermentation	Improvement of malting and mashing, maceration and color extraction of grapes, improvement of primary fermentation, aroma and quality of beer, improvement and clarification of wine, improvement of filtration rate and wine stability, pectinases are used in tea and coffee fermentation
Cellulases and Pectinases	Food	Improvement of maceration, extraction of juices from fruits and vegetables, decreasing the viscosity, clarification of fruit/vegetables juices, improvement in yields of starch and protein, increasing the texture, quality and shelf life of bakery products
Cellulases and pectinases	Pulp and Paper	Co-additive in pulp bleaching, biomechanical pulping, modification of fibre properties, bio characterization of pulp fibers, enzymatic deinking, reduced energy requirement, reduced chlorine requirement, improve fiber brightness, strength properties and pulp freeness and cleanliness, production of biodegradable cardboard, paper towels and sanitary paper
Cellulases and Pectinases	Textile	Bio-stoning of jeans, bio-polishing of textile fibers, improve fabrics quality, improve absorbance property of fibers, softening of garments, improve stability of fabrics, excess dye removal from fabrics, restoration of color brightness
Cellulases and Pectinases	Others	Production of hybrid molecules of various applications, improvement of extraction and quality of olive oil, reducing risk of biomass waste, production of designer cellulosomes

Source: Modified from (Bhat 2000; Kuhad et al. 2011)

The use of cellulase at the commercial level began in early 1970s where the cellulase produced by *Trichoderma* was sold for the research. During mid 1980s cellulases were used for stonewashing denim and for animal feed which was accompanied by the commercial use of cellulase produced by fungal strains mainly *Aspergillus*, *Panicillum* and *Humicola* (Bhat 2000).

Pectinases have been used in many processes such as fruit juice and alcoholic beverage production, wastewater treatment, vegetable oil extraction, tea and coffee fermentation, poultry feed production, textile product production, and paper production (Favela-Torres et al. 2006; Jayani et al. 2005). Mainly, the polygalacturonases are widely used in food industries and facilitate maceration, liquefaction and extraction as well as filtration process of fruits and vegetables juices, processing of wine, coffee and tea fermentation (Hoondal et al. 2002; Soares et al. 2001). *Aspergillus niger* is mainly used for the commercial production of polygalacturonase (Maldonado et al. 2002). There are only a few studies available on the production of polygalacturonase by bacteria (Ahlawat et al. 2008; Jayani et al. 2010; Kashyap et al. 2000).

1.3.1 Cellulases and pectinases pulp and paper industry

In pulp and paper industry, cellulases help to improve the drainage of recycled fibers, de-inking of recycled fibers and help to characterize the fibers by increasing the solubilisation of pulps (Pere et al. 1995; Prasad et al. 1992). They are useful in the manufacture of cardboard paper, soft papers including paper towels and sanitary papers (Hsu and Lakhani 2000; Salkinoja-Salonen 1990).

Pectinases have also been used in pulp and paper industries due to their macerating activity which is helpful for retting of blasts. In paper making process, pectinases depolymerize pectins (Ricard and Reid 2004).

1.3.2 Cellulases and pectinases in textile industry

In textile industry, cellulases are used in bio stoning of denim by removing the excessive dye from the fabric (Belghith et al. 2001). They help in softening the fibers resulting in the faded look of denim. Cellulases have been used in softening and defibrillation of the fibers (Kvietok et al. 1995; Videbaek and Andersen 1993). They are also helpful in softening of the textile without fiber damage.

Pectinases are helpful in the removal of primary cell wall pectin from cotton fiber. The fiber is composed of 95% cellulose and 5% non-cellulosic. The non-cellulosic compounds are found in the primary cell wall are the lattice of pectin (partially methoxylated polygalacturonic acid), protein, and waxes. Pectinases hydrolyse pectin from cotton fiber maintaining the integrity and strength of the fiber. Pectinases with amylases, lipases, cellulase and hemicellulases help to remove sizing agents from cotton by replacing toxic caustic soda. Similarly they also help in bio-scouring which is a novel process for removal of non-cellulosic impurities without cellulose degradation (Jayani et al. 2005).

1.3.3 Cellulases in detergent industry

In the detergents industry, cellulases improve the performance of washing powders by restoring the softness and brightness, uniformity, smoothness of cotton fiber by selectively removal of small and fuzzy fibrils. They are used to produce environmentally friendly detergents as these enzymes help in softening the fibers. They improve the color brightness. The species of *Trichoderma*, and *Aspergillus* are mainly used in detergent industries (Kottwitz and Schambil 2004).

1.3.4 Cellulases and pectinases in food industry

Cellulases and pectinases have a wide application in food biotechnology. Cellulases are helpful for color extraction, clarification, production of fruit purees and quality improvement (Galante et al. 1998). Similarly, pectinases are also added to fruit and vegetable juices after processing for depectinization which helps to increase the concentration of the sugar in juice, increase the storage capacity and avoids microbial contamination (Dey et al 2014; Kashyap et al. 2001). Pectinases reduce the viscosity and increase the absorption in feed by hydrolysis of non-biodegradable fibers or by releasing the nutrients blocked by the fibers (Jayani et al. 2005). A combination of cellulases and pectinases are used in the efficient extraction and clarification of fruit and vegetable juices. They help to decrease the viscosity and increase the yield.

In the brewing industry, cellulases and pectinases are used to improve the brewing process of poor quality barley. They mainly remove the gel formation which causes poor filtration leading to low extract yields. In wine industries, these enzymes help to obtain better skin degradation, improve color extraction, better extraction and improve the quality of the end product (Bhat 2000).

1.3.5 Cellulases and pectinases in animal feed

The animal feed industry plays an important role in agro business. Up to 90% of total feed production is taken by poultry, pigs and ruminants and pet foods and fish farming accounts for 10% (Bhat 2000). Cellulases and pectinases are mainly useful as ruminant feed because they degrade the cereal and cellulosic material to improve the nutritional value and help to supplement the digestive enzymes of animals (Beauchemin et al. 1995; Hoondal et al. 2002). Likewise, in monogastric ruminant feed, these enzymes remove the anti-nutritional factors from grains and vegetables. Further, these

enzymes supplement the digestive enzymes of animals when those are inadequate and improve the overall feed conversion rate (Galante et al. 1998).

1.3.6 Cellulases in bioconversion

Cellulases are being investigated actively in the conversion of lignocellulosic biomass for the production of biofuel. For this the cellulosic biomass is converted to fermentable sugars which can be utilized by microbial cellulase to produce bioethanol and other value added products (Xiong et al. 2014).

1.3.7 Pectinases in degumming/retting of plant bast fibers

Retting is a fermentation process in which pectinase releasing microorganisms decompose the pectin of bark and release fiber. Alkaline pectinase are mainly used in degumming and retting of fiber crops such as jute, flax, hemp, ramie, kenaff (*Hibiscus sativa*) and coir from coconut husks (Kashyap et al. 2001).

1.3.8 Pectinases in waste water treatment

Different industries such as vegetables and fruit processing release pectin containing waste water as by product. When this waste water is pre-treated with pectinases, it is helpful to remove the pectinaceous materials and makes the water more suitable for the decomposition by activated sludge treatment (Jayani et al. 2005).

1.3.9 Pectinases in coffee and tea fermentation

Alkaline pectinases are helpful for coffee and tea fermentation. The fermentation of coffee using pectinase releasing microorganisms removes the mucilage coat from coffee beans and enhances the tea fermentation and foam forming properties of tea (Murthy and Naidu 2011). Since the large scale treatment of tea and coffee with the commercial enzymes is costly, inoculate disregard waste mucilage is used as a source of pectinases during fermentation.

1.3.10 Cellulases and pectinases in purification of plant viruses

Cellulases and alkaline pectinases are helpful to liberate the viruses from the plant tissues and are helpful for pure virus preparations (Jayani et al. 2005).

1.3.11 Cellulases and pectinases in oil extraction

Plant cell wall polymers degrading enzymes have begun to be used in oil preparations. The pectinases with low level of cellulase which are added during grinding of the olives help to release the oil easily during extraction (West 1996). The addition of these enzymes during olive oil extraction helps to increase the extraction with the overall improvement of plant efficiency. The macerating enzymes could be beneficial in the extraction of oils from other agricultural crops.

1.4 Microbial cellulases and pectinases

A large number of microorganisms mainly the fungi and bacteria are capable of producing cellulases and pectinases. Carboxymethyl cellulose and pectin containing agar plates have been used for the qualitative assay of cellulase and pectinase producing bacteria respectively. The ability of microorganisms to produce large amounts of extracellular proteins makes them more suited for the production of these enzymes. The cellulases and pectinases produced by the microorganisms are helpful in the degradation of cellulose and pectin respectively (Vatanparast et al. 2012). Microorganisms are preferred for the production of industrial enzymes because of low production cost.

1.4.1 Cellulase production by microorganisms

Cellulases are produced by many microorganisms in nature (Table 3). These microorganisms generally degrade carbohydrates and cannot use protein or lipids as a source of energy (Lynd et al. 2002). Many bacteria and fungi are the major sources of

microbial cellulases (Watanabe and Tokuda 2010). The fungi, mainly the species of *Trichoderma* and *Aspergillus*, produce extracellular cellulases in comparatively higher amounts than bacteria and have been used industrially (Kumar et al. 2008). While the most commonly studied cellulases producing bacterial strains include the species of *Bacillus*, *Pseudomonas* and *Cellulomonas* and the actinomycetes strains include *Streptomyces* and *Actinomucor* (Sukumaran et al. 2005). The cellulases from these bacteria have a wide application in the conversion of cellulosic biomass to ethanol, organic acids, single cell protein and lipids.

Table 3. Microorganisms producing cellulases

Cellulase producing microorganisms	
Fungi	<i>Aspergillus</i> , <i>Melanocarpus</i> , <i>Penicillium</i> , <i>Trichoderma</i> , <i>Coniophora</i> , <i>Lanzites</i> , <i>Poria</i> , <i>Fomitopsis</i> <i>Phanerochaete</i> , <i>Sporotrichum</i> <i>Trametes</i>
Bacteria	Aerobic bacteria <i>Acinetobacter</i> , <i>Bacillus</i> , <i>Cellulomonas</i> , <i>Paenibacillus</i> , <i>Pseudomonas</i> <i>Salinivibrio</i> , <i>Rhodothermus</i>
	Anaerobic bacteria <i>Acetivibrio</i> <i>Butyrivibrio</i> , <i>Clostridium</i> , <i>Fibrobacter</i> , <i>Ruminococcus</i>
Actinomycetes	<i>Streptomyces</i> , <i>Thermomonospora</i>

Source: Modified from Kuhad et al. (2011)

The production cost of cellulases can be optimized by comparing the production medium and using an alternative carbon source such as municipal solid waste residues which could be an advantage as the enzyme production rate is normally higher by some microbial species (Gautam et al. 2011; Rastogi et al. 2010). Recently, the bacterial cellulases have received more attention over the fungal cellulases because of their high growth rates. Aerobic bacteria produce cellulases in a free form whereas the anaerobic bacteria produce cellulases as a cell associated enzyme complex known as cellulosome which is a multienzyme complex consisting of many subunits (Bayer et al. 2007). The

bacteria isolated from different sources such as sea water (Kim et al. 2009), landfill (Korpole et al. 2011), organic fertilizers and paper mill sludges (Maki et al. 2011) are capable of hydrolysing carboxymethyl cellulose (CMC). Similarly, cellulase producing bacteria have been isolated from some other sources such as the digestive tracts of insects like termite, snail, caterpillar, and bookworm (Gupta et al. 2012) and digestive juices of crab (Bui and Lee 2015). Such bacterial isolates are the ideal candidates for the production of cellulases. Also, the species of *Pseudomonas*, *Bacillus* and *Paenibacillus* strains can degrade lignocellulosic biomass and they have good potential for industrial use (Maki et al. 2012). There are some reports of cellulase production by the species of *Geobacillus*, *Thermobacillus*, *Cohnella*, and *Thermus* which would facilitate development of more efficient and cost-effective forms of the simultaneous saccharification and fermentation process to convert lignocellulosic biomass into biofuels (Rastogi et al. 2010). Sometimes the cellulase production can also be increased by using different substrates like barley and wheat straws which are better suited for the cellulase production by some bacteria like *Geobacillus* sp. T1 (Assareh et al. 2012). Such microorganisms are potential candidates for conversion of agricultural biomass to biofuels. Further, the microorganisms growing on rotting wood samples also produce cellulases in nature and the isolation and identification of such microorganisms might be useful to characterize their enzymes.

1.4.2 Pectinase production by microorganisms

Pectinases are naturally produced by the microorganisms mainly the fungi and bacteria (Table 4). The microbiota present in decomposing fruits (Kumar and Sharma 2012), the gut of insects (Engel et al. 2012) and rumen of sheep (Yuan et al. 2012a) also produce pectinases and help in the digestion of pectin. Microbial pectinases are important in phytopathogenic process, plant microbe symbiosis, and degradation of

pectic substances. Pectolytic enzymes are abundantly produced by fungi and have been used for the industrial applications. The important pectinases producing fungi are the species of *Aspergillus* (Dey et al. 2014; Heerd et al. 2014; Yannam et al. 2014) and the bacteria are the species of *Bacillus* (Gupta et al. 2012; Kashyap et al. 2000; Tepe and Dursun 2014). Most of the fungal pectinases are optimal at the acidic conditions and the bacterial pectinases have optimum activity in alkaline conditions (Kashyap et al. 2001). Some of these microorganisms produce enzymes outside of their cell and release to the medium.

Table 4. Pectinase producing microorganisms

Microorganisms producing pectinases	
Acidic pectinases (PG)	<i>Aspergillus niger</i> CH4, <i>Penicillium frequentans</i> , <i>Sclerotium rolfsii</i> , <i>Rhizoctonia solani</i> , <i>Mucor pusilus</i>
Alkaline pectinases (PG)	<i>Bacillus</i> sp. RK9, <i>Bacillus</i> sp. NT-33, <i>Bacillus polymyxa</i> , <i>Bacillus pumilis</i> , <i>Amucola</i> sp., <i>Xanthomonas compestris</i> , <i>Bacillus</i> No. P-4-N, <i>Bacillus stearothermophilus</i> , <i>Penicillium italicum</i> CECT 22941, <i>Bacillus</i> sp. DT 7, <i>Bacillus subtilis</i> , <i>Bacillus</i> sp. MG-cp-2

Source: Modified from Kashyap et al. (2001)

These microorganisms can use different organic substrates like lemon peel (Rashad et al. 2010) for the extracellular pectinases like polygalacturonase production. In addition, polygalacturonases produced by some bacteria such as *Klebsiella* sp. and *Bacillus licheniformis* KIBGE-IB21 are active in a broad range of pH (Yuan et al. 2012b; Rehman et al. 2015) showing their potential applications in feed and food industry.

1.5 Research rationale

Microorganisms have importance for the large-scale processes of fermentation for the commercial production of industrially important enzymes such as proteases, cellulases and pectinases. Cellulases and pectinases have potential applications for hydrolysing polysaccharides in biorefining industries which are based on agro industrial wastes. However, the cost of the production of these enzymes should be lowered significantly. Further, there is a major problem for industrial production of different value added products by using these enzymes due to the high production cost. Other problems are related with the slow growth rates, long induction period for enzyme expression and the low specific activity of enzyme producing fungi which have been widely used in the industrial sector (Kadam 1996). It is therefore important to screen novel enzyme producing microorganisms, optimize the production and improve the effectiveness of the enzymes.

Several microorganisms including fungi and bacteria produce enzymes for the degradation of cell wall polysaccharides. Still, the selection of a particular strain of interest for the commercial production of enzymes is tedious. Bacterial strain producing commercial enzymes have received preference over the fungal strains as they are easy to grow during fermentation process for enzyme production. Also, the strain improvement techniques are quicker for increasing the yield of production. The isolation and characterization of the bacterial strains is helpful to identify the isolates producing important enzymes including cellulases and pectinases with unique properties and such isolates might be potential candidates for many industrial processes (Gaur and Tiwari 2015; Ghani et al. 2013).

Enzyme characterization is important for its potential application. It is important to develop the methodologies to increase the enzyme stability. The characterization of cellulases and pectinases help to understand the mechanisms of action of these enzymes. So, it will be economically feasible identifying the novel cellulase and pectinase bacterial strains and optimization of enzyme production parameters during fermentation.

1.6 Research objectives

The aim of this study was to isolate bacterial strains capable of producing cellulase and pectinase like polygalacturonase and optimize fermentation conditions for maximum production of these enzymes. *Agave* biomass was also used as substrate for the bioethanol production by the cellulase producing bacteria. The main research objectives are as follows:

1. Isolation and characterization of cellulase and polygalacturonase producing bacteria
2. Optimization of enzyme production by using different parameters
3. Use of biomass as substrates for enzymes production
4. Application of cellulase on biomass for bioethanol production

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CHAPTER II

Characterization of novel cellulase producing bacteria isolated from rotting wood samples

Abstract

Seventeen bacterial isolates were screened for their cellulase activity by carboxymethyl cellulose (CMC) plate assay. The bacterial strain K1 showed the largest depolymerized region in CMC plate assay and was further studied for quantitative cellulase activity. On the basis of 16S rDNA sequence analysis, the strain K1 was found to be *Bacillus* sp. This strain produced the maximum CMCase at pH 6 and 50 °C in presence of peptone (1%) as a source of nitrogen. The CMCase activity was stimulated by Ca²⁺ (2 mM) by 20% over the control. The CMCase activity of this *Bacillus* sp. K1 was highly induced when lactose was used as a source of carbon during fermentation.

Key words: Cellulase, *Bacillus*, optimization

1. Introduction

Due to the high rates of consumption of fossil fuel, there is an increase need for finding a new alternative source of renewable energy. Agricultural biomass is the best alternative source of biofuel (Mussatto et al. 2010; Perlack et al. 2005; Zambare et al. 2011). On average, the biomass of plants contains 30-35% cellulose, 20-35% hemicellulose and 5-30% lignin (Lynd et al. 1999). Cellulose is composed of glucose units joined together by β -1, 4 glycosidic linkages. Hemicellulose is a heterologous polymer of 5 and 6 carbon sugars and lignin is a complex aromatic polymer. Cellulose is the major component of plant cell wall and is one of the most fascinating renewable energy sources (Demirbas 2007; Kim et al. 2006). However, cellulose is not easily amenable to the fermentation which is essential for lignocellulosic biorefineries. For this, the degradation of cellulose to glucose is an important step. This can be achieved by cellulase which is produced naturally by microorganisms mainly bacteria and fungi (Immanuel et al. 2006).

Cellulases are responsible for breaking down the glycosidic linkage in a polysaccharide cellulose (Saha et al. 2006) and hydrolyse cellulose into glucose units. There are 3 types of cellulases which act synergistically. These enzymes are exoglucanase (cleaving β -1,4 glycosidic bonds from chain ends), endoglucanase (randomly cleaving β -1,4 internal linkages) and β -glucosidase (cleaving final β -1,4 linkage of cellobiose or small polysaccharides) (Perez et al. 2002). Most of the cellulases currently used in industrial scale are produced by fungi because of their ability for high enzyme secretion. Bacteria may be considered more ideal candidates for cellulase production as they are fast growing and culturable (Nagendran and Hallen-Adams 2009). Cellulases have several industrial applications including biofuel production, cotton softening, denim finishing, adding to detergents and washing

powders (Cherry and Fidantsef 2003; Vyas and Lachke 2003). The cellulase producing bacteria have been isolated from different sources over the past decades. These sources include soil, decaying wood samples, faeces of ruminants and insect guts (Doi 2008). The present study concentrates the isolation and characterization of efficient cellulase producing bacteria from rotting wood samples which are one of the abundantly available lignocellulosic sources with the possible presence of the cellulase producing bacteria and optimization of the enzyme activity for the possible use in industrial scale.

2. Materials and methods

2.1 Bacterial strains isolation and identification

The samples were collected from the premises of Lakehead University Thunder Bay, ON, Canada. One gm sample of the rotting was suspended in 100 ml of distilled water and was homogenized by vortexing. Serial dilutions of 10X were made by adding autoclaved distilled water. One hundred μ l of each dilution was spread by using standard spread plate method over LB agar plates containing peptone 10 $g\ l^{-1}$, yeast extract 5 $g\ l^{-1}$, NaCl 5 $g\ l^{-1}$ and agar 15 $g\ l^{-1}$. The plates were incubated for 24 h before sampling. From the plates, different colonies of bacteria were selected based on their morphological features like size and color. The pure cultures were streaked out in carboxymethyl cellulose (CMC) agar plates containing CMC 0.5 g, NaNO₃ 0.1 g, K₂HPO₄ 0.1 g, KCl 0.1 g, MgSO₄ 0.05 g, yeast extract 0.05 g and agar 1.5 g w/100 ml.

2.2 Screening for carboxymethyl cellulose activity

The pure bacterial strains were cultured overnight in 7 ml of LB liquid media at 30°C along with *Cellulomonas xylanilytica* and *Escherichia coli* JM109 which were used as a positive and negative controls respectively. Five μ l of each isolate was dropped in a petri plate containing CMC agar medium and then incubated at 30 °C for

48 h. Then, the CMC plates of all the isolates including controls were stained using Gram's iodine solution (2.0 g KI and 1.0 g I, per 300 ml ddH₂O) for qualitative cellulase assay. The iodine solution stains the agar containing CMC forming clear zones in the areas without CMC. These clear zones are known as halo regions which indicate the cellulase activity by the bacteria.

2.3 DNA extraction and amplification of 16S rDNA

The genomic DNA of the cellulase positive isolates was isolated by using ultraclean microbial DNA extraction kit. The extracted DNA was amplified using primers HAD-1 (5'-GACTCCTACGGGAGGCAGCAGT-3') and E1115R (5'-AGGGTTGCGCTCGTTGCGGG-3'). The reaction mixture (25 µl) composed of each primer 1 µl, PCR master mixture 12.5 µl, ddH₂O 8.5 µl and DNA template 2 µl. The PCR was used as follows: primary denaturation 3 minutes at 95 °C, followed by 35 amplification cycles consisting of denaturing at 95°C for 1 minute, annealing for 1 minute at 63 °C, and extension at 72 °C for 1 minute, upon completion of 35 amplification cycles; a final extension step was done at 72 °C for 10 minutes. The PCR products were visualized in 1% gel electrophoresis. The DNA from gel was purified by using Geneaid PCR/Gel purification kit (FroggaBio, Canada) by following the manufacturer's protocol. Then the purified samples were sent for sequencing to Euroffins Genomics (USA).

2.4 Isolates identification and phylogenetic relationship

The sequencing results were inputted to NCBI database (<http://blast.ncbi.nlm.nih.gov/>) for possible identification of bacterial genera using Basic Local Alignment Sequencing Tool (BLAST). The phylogenetic relationship was

analysed by using a sequence alignment program Clustal X (Larkin et al. 2007) and Treeview (Page 1996).

2.5 Bacterial growth and Carboxymethyl cellulase (CMCase) assay

The isolate showing the highest activity in plate assay was further screened for quantitative cellulase assay. Its growth was observed at different time intervals. CMCase activity was determined by measuring the release of reducing sugars from CMC. A modified microplate based assay using 3, 5- dinitro salisalic acid (DNS) method was used to measure the reducing sugar (Miller 1959). For this, 20 μ l of cell free enzyme supernatant was prepared and mixed with 80 μ l solution of 0.5% CMC and 0.5 M citrate buffer of pH 6 and was incubated for 30 min at 50 °C. The reaction mixture was terminated by adding 200 μ l DNS and the mixture was boiled for 5 min. The absorbance was determined at 540 nm.

2.6 Optimization of cellulase

For the optimization of cellulase activity, in most of the experiments 20 μ l of enzyme supernatant was mixed with 80 μ l solution of 0.5% CMC and 0.5 M citrate buffer and the mixture was incubated for 30 min at 50 °C

2.6.1 Effect of incubation period in cellulase production

The culture tubes containing minimal salt medium (NaNO_3 0.1 g l^{-1} , K_2HPO_4 0.1 g l^{-1} , KCl 0.1 g l^{-1} and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 g l^{-1}) and 1% CMC were cultured and 1 ml of sample was harvested on each day starting from the first day of inoculation. The cell free supernatant was used for enzyme assay.

2.6.2 Effect of pH and temperature on cellulase activity

The CMCase activity was measured at different acidic, neutral and basic pH. Similarly, the effect of temperature on cellulase activity was carried out at different temperatures from 30 °C to 70 °C.

2.6.3 Effect of metal ions and surfactants

The effect of different metal ions, Ca^{2+} , Co^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} in their chloride salts, on the activity of cellulase was determined by performing the CMCase assay in the presence of these metal ions (2 mM) at 50 °C for 30 min. For this assay, the reaction mixture contained 20 μl enzyme supernatant, 10 μl metal ion, 70 μl 0.5 M citrate buffer (pH 6) and 1% substrate (CMC). Further, different concentrations of the most effective metal ion was used. The effects of detergents Sodium Dodecyl Sulphate (SDS, 10 mM) and Triton X-100 (10%) were observed on the CMCase activity.

2.6.4 Effect of different nitrogen sources on cellulase production

Nitrogen sources (0.5% w/v) used were yeast extract (YE), peptone, urea and ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$ in the enzyme production medium to determine their effects in enzyme production. For determining the best concentration of the most effective nitrogen source, the activity was tested under the same optimal pH and temperature.

2.6.5 Effect of carbon sources on cellulase production

Various carbon sources (1% w/v) were used to determine the effect of carbon source on cellulase production medium. The carbon sources used were CMC, glucose, sucrose, sorbitol, lactose, mannose and galactose.

2.7 SDS-Polyacrylamide gel electrophoresis (PAGE)

For the determination of molecular weight of the cellulase from the isolated bacterial strain K1, the crude enzyme was first incubated at 50 °C for five minutes and was run along with standard protein markers in 10% SDS PAGE according to Laemmli (1970). For this, the electrophoresis was carried out with the constant supply of 200 V current. The gel was stained with Coomassie Brilliant Blue R-250 solution for one hour and destained with decolor buffer for proteins and markers bands. The SDS gel containing 0.25% CMC was used for the detection of cellulase activity and was washed with Triton X-100 for 15 minutes, then it was incubated at pH 6 buffer at 50 °C for 30 minutes. Following this, the gel was washed and stained with 0.1% Congo red for 30 minutes and destained with 1M sodium chloride solution for zymogram analysis.

2.8 Statistical Analysis

All the experiments were performed in triplicates and the results are expressed in terms of mean \pm SD (standard deviation). The statistical analysis of data was performed to test the significant difference by one way analysis of variance (ANOVA) followed by Tukey's HSD test ($p < 0.05$) using Statistical Package for the Social Sciences (SPSS) system.

3 Results and Discussion

3.1 Isolation and identification

Sixty bacterial samples were collected from different locations around Thunder bay, Ontario, Canada. Seventeen strains showed cellulase activity in CMC agar plate assay (Fig. 1). This method of isolation was found easy for preliminary screening of cellulolytic bacteria. The strains were compared with a cellulase producing positive control (*Cellulomonas xylanilytica*) and negative control (*E. coli* JM109) with no

cellulase activity (Maki et al. 2011). The bacterial strain K1 showed the largest diameter of halo region and was selected for further enzyme assay (Fig 1). The morphological examination showed the colonies of the strain K1 as a rough opaque and grey. The other bacterial colonies also exhibited similar morphological features. As there are wide varieties of cellulase producing bacteria in the environment, their morphological features make the isolation of bacteria easier from different sources.

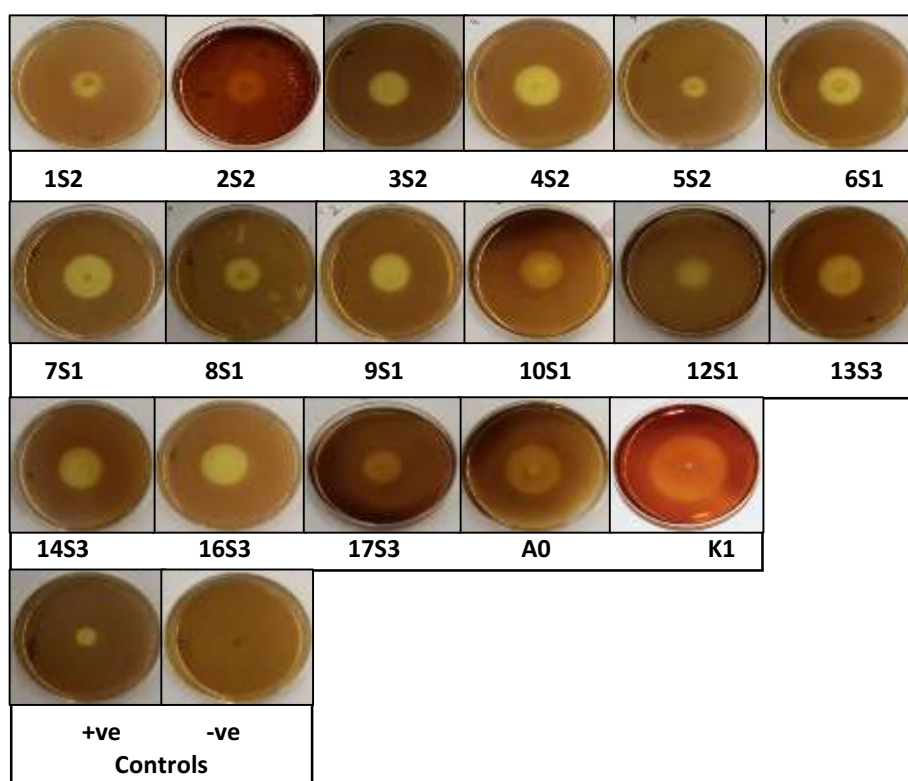


Figure 1 Seventeen cellulase-producing isolates and a positive and negative control, *C. xylanilytica* and *E. coli* JM109.

3.2 DNA extraction and amplification of 16S rDNA

The genomic DNA of all the seventeen isolates was successfully extracted. The PCR primers successfully amplified 16S rDNA fragments. 1% agarose gel showed the clear bands of about 800 bp.

3.3 Isolates identification and phylogenetic analysis of 16S rDNA sequences

The sequences of all the 17 isolates were analysed by using nucleotide blast of NCBI database. The genera of 17 isolates were identified on the basis of DNA sequences homology. The isolates are related to *Bacillus* (12), *Pseudomonas* (3) *Rahnella* (1) and *Buttiauxella* (1). The sequence for K1 was successfully uploaded to NCBI gene bank database (Accession no. KP987117).

For the phylogenetic analysis, the sequencing results of all the seventeen cellulase producing bacterial isolates were aligned using ClustalX UPGMA algorithm. The sequences were uploaded into TreeView for phylogenetic relationship analysis (Fig. 2). The phylogenetic analysis revealed that the isolates belong to two groups Firmicutes and Proteobacteria. The *Bacillus* strains are related to Gram positive Firmicutes and the strains *Pseudomonas*, *Rahnella* are *Buttiauxella* related to Gram negative Proteobacteria. Both the groups of bacteria can degrade the cellulosic materials.

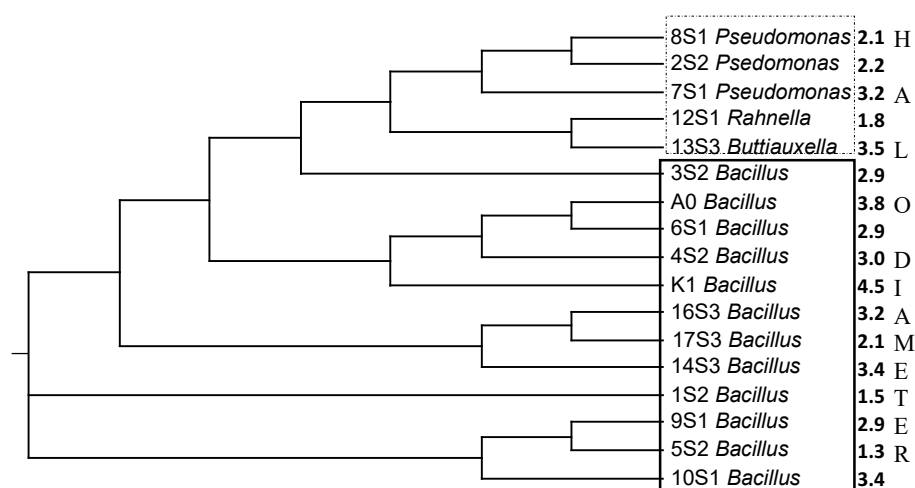


Figure 2 Phylogenetic tree depicting the evolutionary relationships between the seventeen cellulase positive bacterial isolates (displayed using Tree view) and halo diameter (cm). The isolates outlined in black belong to Firmicutes and those dashes outlined isolates belong to Proteobacteria. The numbers represent the halo diameters produced by the cellulase producing bacteria in CMC agar plates.

3.4 Growth of strain K1 and enzyme production

A time course of the bacterial strain and enzyme production was performed over a period of 120 h. The strain K1 showed maximum growth after three days of incubation. Also, the cellulase yield reached a maximum at 72 h of incubation (Fig. 3) which was significantly different to the cellulase production at 24 h, 96 h and 120 h. The fermentation period is an important factor for enzyme production by microorganisms (Gautam et al. 2011). Similar results of maximum production of cellulase at 72 h of incubation were found by other researchers. The *B. pumilis* EWBCM1 and *B. sp.* B21 showed maximum endoglucanase after 72 h incubation (Amritkar et al. 2004; Shankar and Isaiarasu 2011). However, this enzyme production time was different from other researchers who reported the maximum endoglucanase after 24 h in *Pseudomonas sp.* HP207 (Sheng et al. 2012) and *Pseudomonas fluorescens* NCIB (Dees et al. 1994), 96 h in *Bacillus circulans* and *Bacillus subtilis*, 142 h incubation for *Clostridium cellulolyticum* (Guedon et al. 2002). The *Bacillus* strains produce cellulase at different time intervals and are regarded as the important cellulase producers in enzyme industry (Priest 1977).

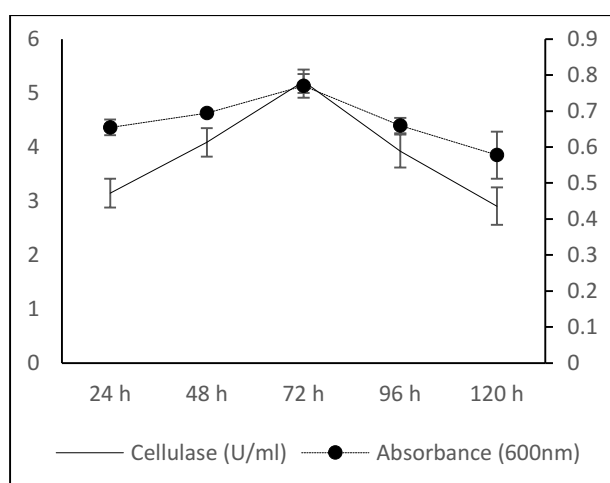


Figure 3 Growth of strain K1 and CMCase production by *Bacillus sp.* K1

The CMCase activity of strain K1 was compared with positive control *Cellulomonas xylanilytica* and negative control *E. coli* JM 109. The CMCase activity of strain K1 was 5.21 ± 0.21 U/ml (Fig. 3) whereas this activity for *C. xylanilytica* was 2.28 ± 0.51 U/ml and *E. coli* JM 109 exhibited no CMCase activity. One unit (U) of cellulase activity is defined as the amount of enzyme necessary to release 1 μ mol reducing sugar per minute per ml. This enzyme activity of strain K1 was found higher than those of widely studied bacteria and some fungi, which have received wide attention for commercial production of cellulase (Kang et al. 2004). Sheng et al. (2012) reported endoglucanase activity by *Pseudomonas* sp. under optimized conditions to be 1.432 U mL^{-1} . Under different nutritional and environmental factors, the endoglucanase activity of *Bacillus pumilus*, *Aspergillus niger*, and *Trichoderma harzianum* Rut-C 8230 did not exceed 1.0 U mL^{-1} (Ariffin et al. 2008; Kocher et al. 2008; Kotchoni and Shonukan 2002; Narasimha et al. 2006). Similarly, CMCase activity was only 0.12 U/ml by *Bacillus* sp.(Rastogi et al. 2010) and 0.8U/L by *Geobacillus* sp.(Abdel-Fattah et al. 2007).

3.4.1 Effect of pH and temperature on cellulase activity

The CMCase activity of strain K1 was found maximum at pH 6 (Fig. 4A) which was significantly different to other pH tested during the experiment ($p < 0.05$). The enzyme showed significant decrease after this pH retaining 38% of its activity at pH 8. Similar result was also reported in *Bacillus* sp. CH43 (Robson and Chambliss 1989). A pH of 6.5 was found to be optimal in other *Bacillus* strains (Kim et al. 2009; Robson and Chambliss 1989). The *Bacillus* strains CH43 and HR68 showed stable cellulase activity in pH 6-8 (Mawadza et al. 2000).

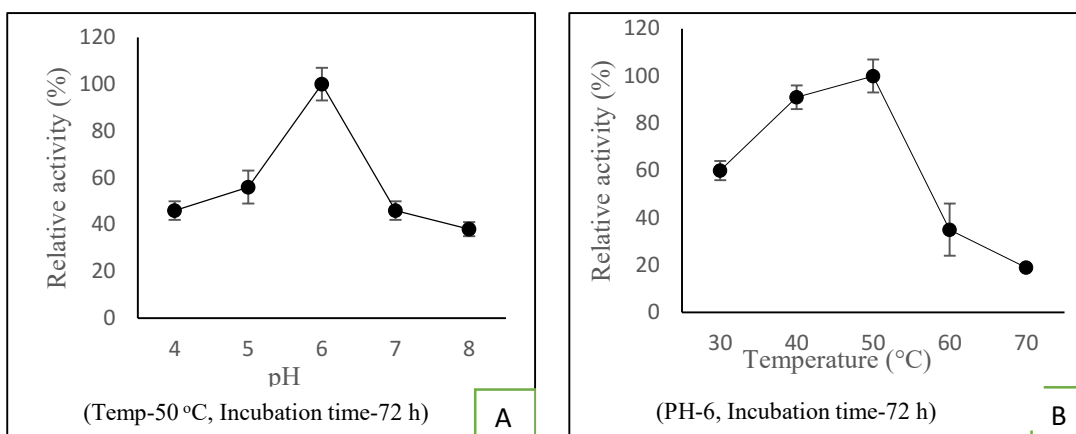


Figure 4 Effect of pH (A) and temperature (B) on CMCase activity by *Bacillus* sp. K1

Microbial cellulase activity has been influenced by temperature. The optimal temperatures are different in different bacteria. The bacterial strain K1 showed cellulase activity from 30 °C to 70 °C. The maximum enzyme activity was found at 50 °C and this activity was significantly different to the CMCase activity at 30 °C, 60 °C and 70 °C ($p < 0.05$). At 70 °C, the enzyme showed 19% of its relative enzyme activity (Fig. 4B). Similar results have been found in other *Bacillus* spp. (Kim et al. 2009; Lee et al. 2008; Sadhu et al. 2014).

3.4.2 Effects of Metal ions and surfactants on cellulase activity

The CMCase activity by Ca^{2+} was significantly different ($p < 0.05$) to control, other metal ions and detergents used in the experiment (Fig. 5A). Fu et al. (2010) also reported that Ca^{2+} , Mg^{2+} and Mn^{2+} had positive effect on endoglucanase activity of *Paenibacillus* sp. BME-14. Ca^{2+} ions have been found essential for enhancing the substrate binding affinity of the enzyme (Mansfield et al. 1998). Maximum enzyme activity was observed at 2 mM Ca^{2+} (Fig. 5B).

The cellulase produced by strain K1 was not tolerant to the common detergents SDS and Triton X-100. The enzyme was reduced to about 60% while using these surfactants (Fig. 5A) which was significantly lower than the control ($p < 0.05$). It might

be because of the interaction of detergents with the hydrophobic group of amino acids. The surfactant like SDS has been found to reduce the endoglucanase activity (Aygan and Arikan 2008).

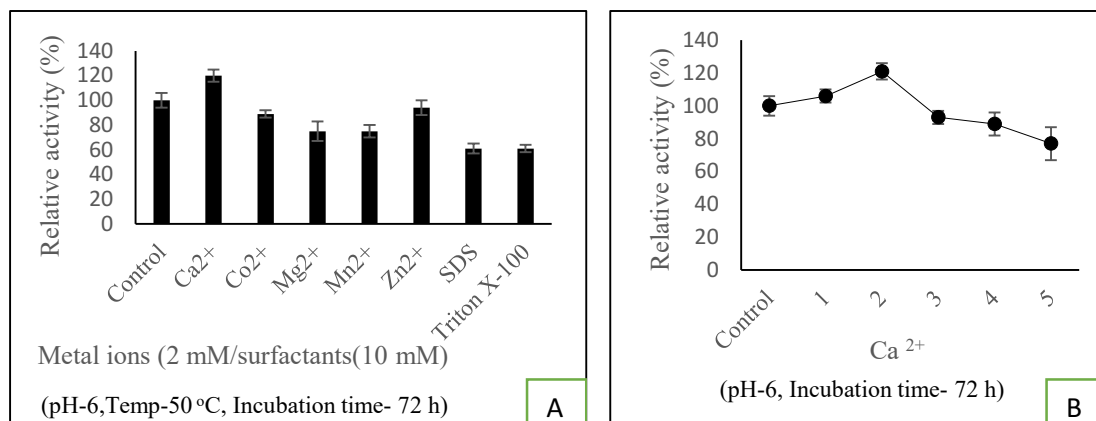


Figure 5 Effect of metal ions and surfactants (A) and Ca²⁺ on CMCase activity (B) by *Bacillus* sp. K1

3.4.3 Effect of different nitrogen sources in culture medium during cellulase production

The production of cellulase is sensitive to source of nitrogen. The maximum CMCase activity was found by using peptone as a source of nitrogen (Fig. 6A) which was significantly different to ammonium sulphate and urea ($p < 0.05$). The *Bacillus* strain could utilize the source of organic nitrogen. The reduction in the production of inorganic nitrogen source might be due to the medium acidification which affected the

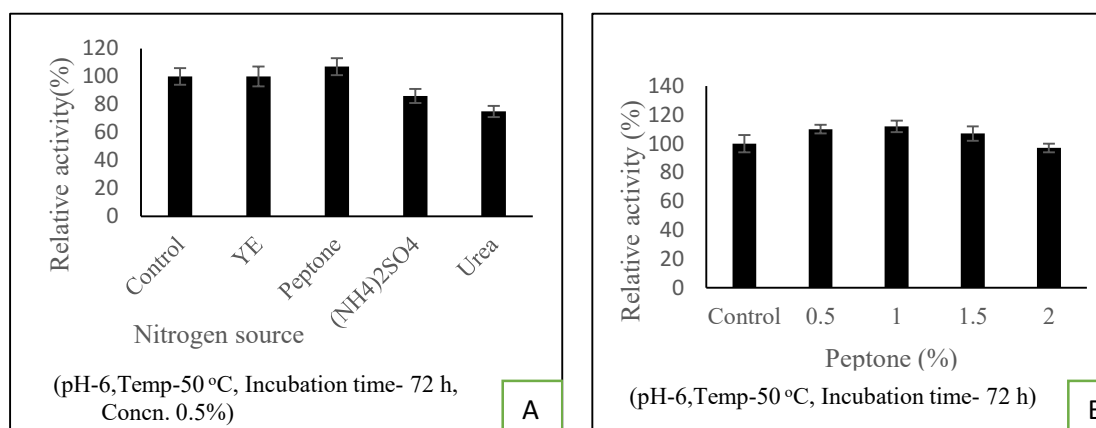


Figure 6 Effect of nitrogen source (A) and peptone concentration (B) on CMCase production by *Bacillus* sp. K1

cellulase production. The *Bacillus* sp. isolated by Yang et al. (2014) and Bairagi et al. (2007) showed similar results of organic nitrogen source for cellulase production. However, the *Bacillus subtilis* could utilize both the inorganic and organic nitrogen source for cellulase production (Acharya and Chaudhary 2011). The use of 1% of peptone enhanced the production of cellulase by 12%. On increasing the concentration of peptone after 1%, the enzyme activity was decreased significantly (Fig. 6B).

3.4.4 Effect of different carbon sources in culture medium during cellulase production

In this experiment, the results showed that the strain K1 could utilize various carbon sources in the production medium and the use of lactose in the culture medium showed significantly different and higher CMCase activity ($p < 0.05$) to that of other source of carbon used in the experiment (9.96 ± 0.23 U/ml) (Fig. 7A). While using different concentrations of lactose, the maximum cellulase was produced when 1% lactose was used in the medium (Fig. 7B). Since the cellulase is an inducible enzyme, the production of enzyme is enhanced sometimes by some sources of carbon in the medium.

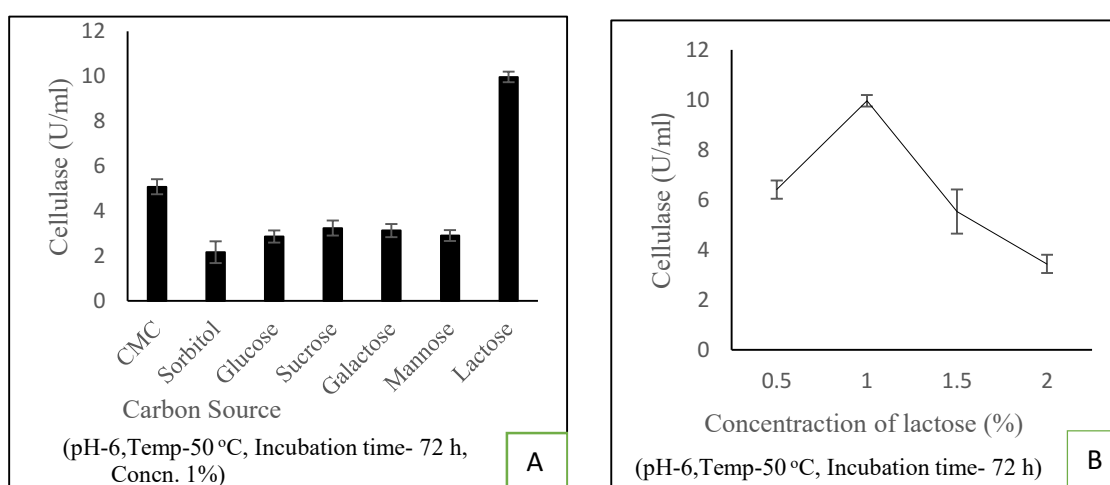


Figure 7 Effect of carbon source (A) and lactose concentration (B) on CMCase production by *Bacillus* sp. K1

Lactose in the production medium was quickly taken up by the isolated *Bacillus* strain and the CMCase was produced. It might be due to the lactose induced enzyme activity or increased the rate of penetration through the cell membrane (Miyamoto et al. 2000). Also, lactose enhances the cellulase yield by stimulating the secretion of various proteins with cellulase. Other researchers also reported the maximum CMCase production by using lactose as a source of carbon by *Microbacterium* sp. (Sadhu et al. 2011), *Aspergillus hortai* (El-Hadi et al. 2014), *Trichoderma reesei* (Karaffa et al. 2006).

3.5 SDS- PAGE and zymogram analysis

Based on the zymogram which was run under the conditions of SDS-PAGE, the molecular weight of the crude cellulase was estimated ~36 kDa (single band of K1, Fig. 8). This is similar to the findings of many researchers who reported the molecular weight of cellulases from 37-43 kDa in *Bacillus* species (Bischoff et al. 2006; Hakamada et al. 2002; Ozaki and Ito 1991).

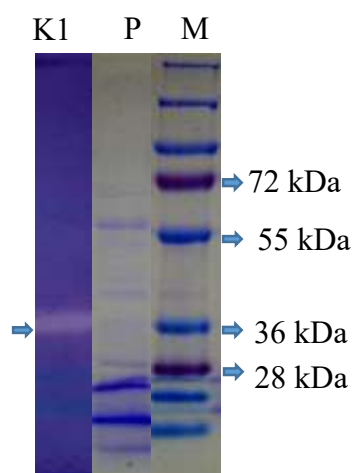


Figure 8 SDS-PAGE and zymogram of crude cellulase enzyme. (K1- cellulase zymogram, P- protein in supernatant and M- marker; Based on the gel, the molecular wt. of the enzyme was estimated about 36 kDa)

However, this molecular weight of cellulase was lower than the other species of *Bacillus* from which cellulases had molecular weight of 53–78 kDa (Christakopoulos et al. 1999; Li et al. 2006; Okoshi et al. 1990).

4. Conclusions

Seventeen cellulase producing bacterial isolates were isolated from different rotting wood samples. The isolate K1 produced higher cellulase in plate assay than other isolates. Similarly, the quantitative CMCase activity of this strain (5.21 ± 0.21 U/ml). On the basis of 16S rDNA sequence analysis, the strain K1 was found to be *Bacillus* sp. This strain produced maximum CMCase at pH 6 and 50 °C after 72 h of incubation. The cellulase produced by this strain was enhanced by Ca²⁺ ions. In the production medium, 1% peptone enhanced the cellulase production by 12% over the control. Similarly, lactose induced the CMCase nearly doubling the enzyme production (9.96 ± 0.23 U/ml). So, this strain is of particular interest for enzyme induction for producing maximum cellulase which might be valuable for biorefining industries. Based on SDS-PAGE analysis, the molecular weight of the cellulase was found ~36 kDa.

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CHAPTER III

Two *Bacillus* species isolated from rotting wood samples are good candidates for the production of bioethanol using *Agave* biomass

Abstract

The biorefining of crop and plant organic matter represents a promising route to produce renewable fuels and bioproducts. *Agave americana* is a xerophytic plant. The high contents of cellulose and hemicellulose with low content of lignin make *Agave americana* an ideal candidate to produce value-added products. In this study, two different cellulase producing *Bacillus* strains, isolated from rotting wood samples, were incubated and cultivated to examine their ability to decompose *Agave* and produce ethanol. The results showed the transparent zones called halos on the plates containing *Agave* as the sole carbon source after iodine staining for these two isolates and positive control *Cellulomonas xylanilytica* ; while, no halo was detected for negative control; *Escherichia coli* BL21. The *Bacillus* species K1 and A0 displayed hydrolysis ability greater than that of positive control based on halo diameter. Moreover, the quantitative ability to decompose agave was studied for the same two bacterial strains using minimal salt media containing 5% *Agave* biomass. Dinitrosalicylic acid (DNS) method was used to detect cellulase and reducing sugars. Ethanol was detected by using micro-dichromate method. The results showed that both of the bacterial strains produced ethanol using lignocellulosic biomass of *Agave*.

Key words: *Agave*, lignocellulosic biomass, cellulase, bioethanol

1. Introduction

With the exhaustion of non-renewable fossil fuels leading to environmental pollution and energy crisis, there is a demand for new renewable sources of energy. Biofuels are the renewable source of energy which are environmentally friendly with low carbon dioxide emission.

Bioethanol is the biofuel mostly useful for transportation worldwide. It can be produced from different raw materials such as simple sugars, starch, agricultural products, and lignocellulosic biomass. Recent production of bioethanol is dependent on starch and sugars from existing food crops (Smith 2008). Although it might be beneficial to use renewable plant materials for bio-fuel, the use of crop residues and other biomass for bio-fuels raises many concerns about major environmental problems such as food shortages and serious destruction of vital soil resources (Pescatori 2010). This makes the availability of raw materials one of the major problems associated with the bioethanol production. To overcome this problem, lignocellulosic biomass constitutes the world's largest bioethanol renewable source. The production of bioethanol from lignocellulosic biomass is one way for reducing the consumption of petroleum oil and environmental pollution. Also, the lignocellulosic biomass is the most promising feedstock considering its great availability and low cost. However, the large-scale commercial production of fuel bioethanol from lignocellulosic materials has not been implemented. The main reason for this is the production cost of bioethanol from lignocellulose is too high because of high production cost of enzymes.

Agave can grow in a dry land with limited water supply and the biofuel produced from *Agave* biomass has very low CO₂ emissions (35 g/J); whereas there is higher CO₂ emission from corn based biofuel (85 g/J) (Yan et al. 2011). *Agave* consists of natural

fibers which can be degraded to a large number of bioproducts and value added products like bioethanol and xylitol (Xiong et al. 2014). So, this plant has potential application for the bioethanol production (Fig. 1). Its fibers are rich source of cellulose (68%) and other components are hemicelluloses (15%), lignin (5%), wax (0.26%), and moisture (8%) (Mylsamy and Rajendran 2010). Cellulose is a polysaccharide formed by D- glucose units linked together by β 1, 4 glycosidic bonds and is insoluble in water but can be hydrolysed by acid, hemicellulose is composed of mainly the pentoses and hexoses and is not soluble in water but soluble in alkali and easily hydrolysed in diluted acids. Lignin is a complex phenolic polymer and is not soluble in water. The main role of lignin is to provide structural support, prevent oxidation and protect the cell against the microbial invasion.

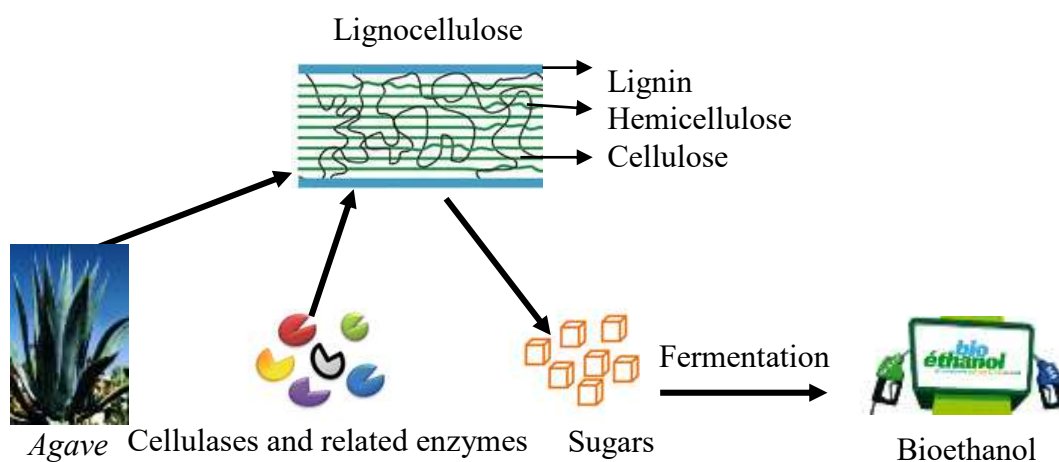


Figure 1 Diagrammatic representation of bioethanol production from *Agave* lignocellulosic biomass

For the production of bioethanol from lignocellulosic biomass, the cellulose is typically hydrolysed by an enzyme called cellulase. Microorganisms mainly the fungi and bacteria are the good candidates for lignocellulosic biomass degradation. Fungi such as *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium* are widely used to produce cellulases (Mani et al. 2002). Bacteria belonging to *Clostridium*, *Bacillus*,

Thermomonospora and *Ruminococcus* can produce cellulases effectively as well (Sun and Cheng 2002).

Bacteria offer several benefits over the fungi for the degradation process of biomass as they have high growth rates as compared with fungi and other microorganisms. Also, bacteria can adapt to different types of environmental conditions in a wide range of pH and temperature. They can also be genetically engineered to increase the catalytic activity for the enzymes degrading lignocellulosic biomass (Pandey et al. 2013). This study aims the production of bioethanol from low high cellulose and low lignin containing *Agave* biomass by using two *Bacillus* strains.

2. Material and methods

2.1 Chemicals and bacterial strains

All the chemicals used in this research were of analytical grade. *Agave americana* biomass (untreated) was obtained our lab at Lakehead University. We previously isolated seventeen cellulase producing bacterial strains from rotting wood samples around Thunder Bay, Ontario, Canada. The cellulase production of the isolate K1 (NCBI Accession no. KP987117) and its molecular weight which was estimated in our previous research, was now compared with the bacterial isolate A0 (NCBI Accession no. KP974676) which was isolated in our previous research. Both of the *Bacillus* spp. were tested for their activity to degrade *Agave* biomass. *Cellulomonas xylanilytica* and *Escherichia coli* BL21 were used as positive and negative controls respectively.

2.2 Screening of cellulase activity

For the screening of cellulase producing activity of bacterial strains, each strain was grown with positive and negative controls in 5 ml LB broth at 30 °C at 200 rpm separately. Five microliters of each sample was inoculated on agar plates which contain 5.0 g^l⁻¹ *Agave*, 1.0 g^l⁻¹ NaNO₃, 1.0 g^l⁻¹ K₂HPO₄, 1.0 g^l⁻¹ KCl, 0.5 g^l⁻¹ MgSO₄, 0.5 g^l⁻¹ yeast extract, 1.0 g^l⁻¹ glucose and 15.0 g^l⁻¹ agar. After incubating all the plates for 48 h at 30 °C, the plates were checked with Gram's iodine solution (Kasana et al. 2008). The diameter of halo region (D) and bacterial colony (d) were measured to show the hydrolysis ability which can be expressed as (D/d)².

2.3 Determination cellulase activity and reducing sugar

For the determination of reducing sugar from the *Agave* biomass degradation, the bacterial strains were grown overnight in LB broth medium. Then 200 µl of the overnight LB grown bacteria were transferred to 50 ml Dubois salt medium containing 0.1 g^l⁻¹ NaNO₃, 0.1 g^l⁻¹ K₂HPO₄, 0.1 g^l⁻¹ KCl, 0.05 g^l⁻¹ MgSO₄ and 5% *Agave* biomass. The bacterial strains were incubated at 30 °C, shaking 200 rpm for seven days. The reducing sugars and cellulase activity were detected by using 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). For this, 1ml of bacterial culture was harvested from each samples. It was centrifuged for 2 min at 15000 g. Carboxymethyl cellulose (CMC) was used as substrate for cellulase activity. Briefly, 20 µl of enzyme supernatant was added to 80 µl of substrate buffer (0.5% CMC in 0.05 M potassium phosphate buffer, pH 6.0) and incubated at 50 °C for 30 min. The reducing sugar released as glucose was determined. Microtitre plate was used for recording the absorbance at 540 nm using Epoch microplate spectrophotometer (BioTek). After seven days, the bacterial strain's survival was confirmed by the drop plate method.

2.4 SDS-Polyacrylamide gel electrophoresis (PAGE)

The molecular weight of cellulase was confirmed by using SDS- PAGE according to the method of Laemmli (1970) using Bio-Rad electrophoresis apparatus. The protein marker and enzyme were allowed to run simultaneously to determine the molecular weight of the enzyme. After completion of the electrophoresis, Coomassie Brilliant Blue R-250 was used to stain the gel. The gel containing 0.25% CMC was used for detection of cellulase activity. The gel was then washed with 2% Triton X-100 for 30 min. Then, it was transferred in pH 7 and incubated at 50 °C for 30 min. After that the gel was stained with 0.1% Congo Red solution and the over staining was removed with 1M NaCl to visualize the clear bands of cellulase activity.

2.5 Ethanol determination

The bacterial strains were grown for 7 days in 50 ml of Dubois minimum salt medium with 5% *Agave* biomass at 30 °C, shaking at 200 rpm. One ml aliquot of the cultured bacteria was centrifuged for 1 min at 17000 x g. The supernatant was used for bioethanol analysis. The samples were then analysed by using micro-dichromate method. In this method, there is complete oxidation of ethanol by dichromate in the presence of sulphuric acid with the formation of acetic acid. Dichromate Cr_2O_7 is yellowish in color and the reduced chromic product (Cr^{3+}) is intensely green. A standard curve of ethanol was made with different concentrations of ethanol the absorbance of the samples was read at 584 nm (Caputi et al. 1968).

2.6 Morphology of *Agave* fiber

Scanning Electron Microscope (SEM) was used to observe the morphological changes of *Agave* fiber. The samples treated with bacterial strains up to 7 days were collected along with the control (samples without bacterial treatment). Each sample was

washed with 0.1M phosphate buffer and dehydrated with ethanol. After this, the samples were dried at room temperature and coated with gold in a Denton-DeskII sputter coater (Denton Vacuum USA, Moorestown, NJ). The samples were observed on SEM (Hitachi SU-70, Japan).

2.7 Statistical analysis

All the experiments were performed in triplicates and the results are expressed in terms of mean \pm SD (standard deviation). The statistical analysis of data was performed by one way ANOVA followed by Tukey's HSD test ($p < 0.05$) using SPSS system.

3. Results and discussion

3.1 Cellulase screening using Agave as a source of carbon

In this research, both the *Bacillus* strains showed the area of depolymerisation which proved their ability for hydrolysis of *Agave* biomass (Fig. 2). The negative control did not have any cellulase activity so there was no halo region. As shown in table 1, the hydrolysis ability values for strains K1 and A0 were 19.64 ± 3.98 and 14.32 ± 0.66 respectively; both of the values were higher than the positive control after 48 h of incubation. This showed that both the bacteria have better ability than *C. xylanilytica* for hydrolysis of *Agave* biomass. As reported by other researchers, *Bacillus* strains have potentiality for the degradation of lignocellulose (Howard et al. 2004).

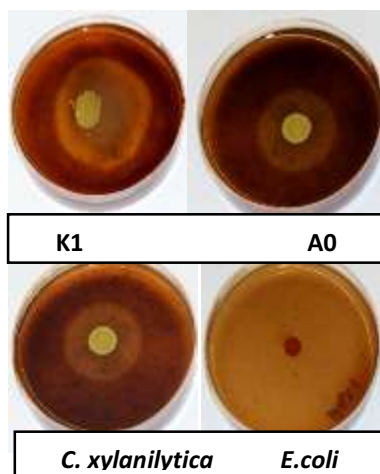


Figure 2 *Agave* plate assay

Table 1. Hydrolysis ability of the bacterial isolates with positive (+ve) and negative (-ve) control

Bacterial isolates	Halo diameter (D, cm)	Colony diameter (d, cm)	Hydrolysis ability $(D/d)^2$	Species
K1	4.8±0.4	1.1±0.2	19.64±3.98	<i>Bacillus</i> sp. K1
A0	3.4±0.3	0.9±0.1	14.32±0.66	<i>Bacillus</i> sp. A0
+ve control	3.3±0.3	0.9±0.1	13.44±0.55	<i>C. xylanilytica</i>
-ve control	-	0.7±0.1	-	<i>E. coli</i> . BL21

3.2 Cellulase activity and reducing sugar production

Since, these bacterial isolates showed maximum growth after three days of incubation (Fig. 3). The cellulase activity was assessed at 72 h of incubation by growing the bacteria in minimal salt medium with CMC and with *Agave* biomass respectively. The CMCase activity of strains was compared with positive control *C. xylanilytica* and negative control *E. coli* BL21 (Fig. 4A). The CMCase activity of strain K1 was 5.21 ± 0.21 U/ml and A0 was 4.3 ± 0.25 U/ml. Similarly, while using the *Agave* biomass as a source of carbon during fermentation the CMCase activities of K1 and A0 were 3.82 ± 0.24 U/ml and 3.5 ± 0.12 U/ml respectively (Fig. 4B). The enzyme activity of these

isolates was found higher than those of the most widely studied bacteria and fungi, which have received wide attention for commercial production of cellulase (Kang et al. 2004).

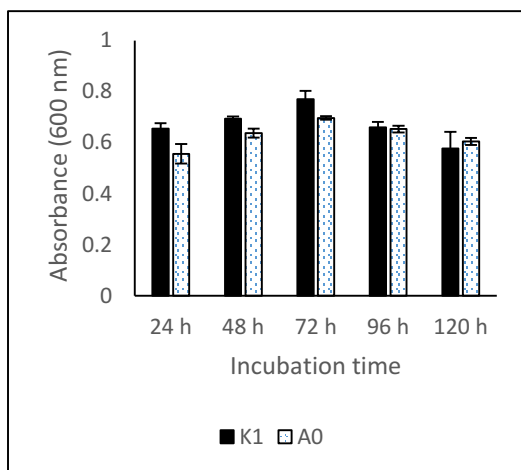


Figure 3 Bacterial growth in CMC minimal salt liquid medium

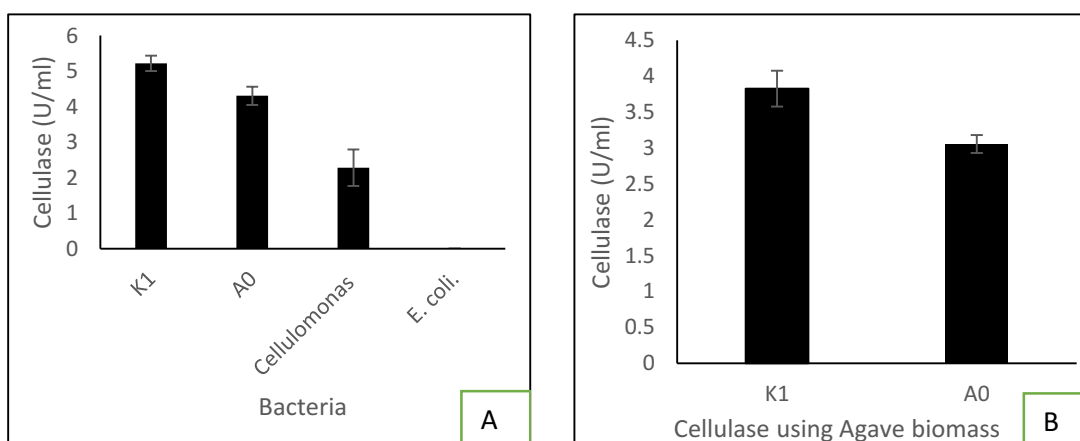


Figure 4 CMCase activity *Bacillus* sp. K1 and *Bacillus* sp. A0 using CMC (A) and *Agave* biomass (B) in production medium

No detectable reducing sugars were observed after 7 days of incubation. It might be due to the fact that the production of bioethanol requires consumption and conversion of sugars during the bacterial growth. The production of reducing sugar might have been decreased with the increase in incubation period which could be due to the consumption and conversion to other chemicals by these *Bacillus* strains. Further, the consumption of reducing sugars by bacteria prevented the inhibition effect of these sugars on enzymatic hydrolysis and ultimately end-product production, producing

ethanol. After seven days of incubation, the survival rate of the both the strains was 100%.

3.3 SDS-Polyacrylamide gel electrophoresis (PAGE) and zymogram analysis

The molecular weight of the crude cellulases produced by K1 and A0 were estimated ~36 kDa ~40 kDa respectively (Fig. 5). Many researchers reported that the molecular weight of cellulases produced by *Bacillus* species ranges from 37-43 kDa (Bischoff et al. 2006; Hakamada et al. 2002; Mawadza et al. 2000; Ozaki and Ito 1991). However, other reports show the molecular weight of cellulases by other species of *Bacillus* to be 53–78 kDa (Christakopoulos et al. 1999; Li et al. 2006; Okoshi et al. 1990).

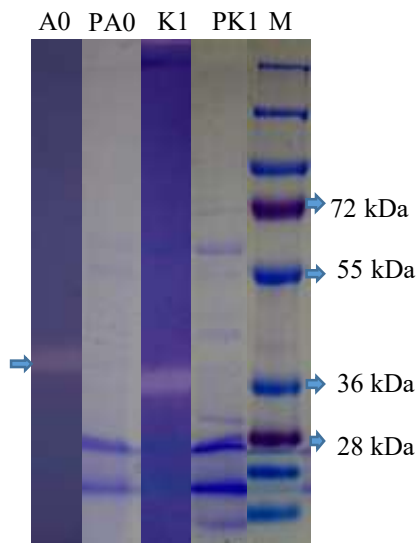


Figure 5 SDS-PAGE and zymogram of crude cellulase enzyme. (A0- cellulase by strain A0, PA0- protein in supernatant, K1- cellulase by strain K1, PK1- protein in supernatant and M- marker; Based on the gel, the molecular wt. of the enzyme was estimated about ~36 kDa for K1 and ~40 kDa for A0)

3.4 Production of ethanol by degradation of Agave biomass

The microorganisms require nutrients for energy generation and enzyme production. The source of lignocellulosic biomass functions as the main source of carbon. The production of ethanol is influenced by different factors such as carbon

source, nitrogen source, culture conditions etc. (Tsigie et al. 2013). The results showed that both the strains produced ethanol efficiently. The maximum bioethanol was produced by strain K1 0.435 g/g of *Agave* fiber and the strain A0 produced 0.397 g/g ethanol at the 4th day of incubation which was significantly different to its production on the other days by both the strains. The ethanol yield was decreased significantly after 4 days of incubation by both the bacterial isolates (Fig. 6). It might be due to the toxic effect of ethanol to the bacteria or that they are metabolizing it to another product. Both the *Bacillus* strains showed a good yield of ethanol from *Agave* biomass without pre-treatment. Further, the strains were able to convert the reducing sugar to ethanol from the first day of incubation to day seven. The enzymatic hydrolysis gives better ethanol yields than thermal acid hydrolysis after fermentation (Villegas-Silva et al. 2014). During enzymatic hydrolysis, if the biomass is pretreated, the production of ethanol is always higher. Also, the hydrolysis ability and ethanol tolerance of microbial strains is different.

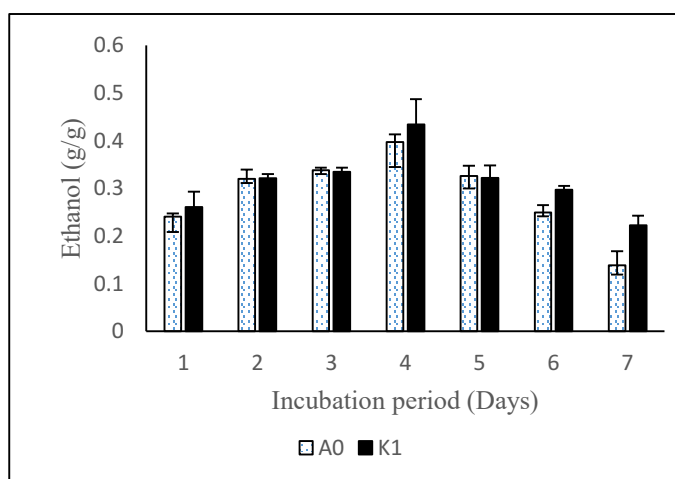


Figure 6 Ethanol yield from degradation of *Agave* biomass expressed in Ethanol (g/L)/ (50 g *Agave* fiber per liter).

The development of low cost and high efficiency substrates like *Agave* in industrial scale is very important. However the conversion of *Agave* lignocellulosic biomass to bioethanol and other value added products is limited by several factors such

as the complexity in pre-treatment, low conversion efficiency from pentose to ethanol, and high production cost of the enzyme. In this research both of the cellulase producing *Bacillus* strains could degrade the untreated *Agave* biomass and produced ethanol. The use of untreated biomass could eliminate the pre-treatment step. Ultimately, it lowers the production cost of ethanol by combining the hydrolysis and fermentation steps together. Similar reports of ethanol production by *Bacillus* strains from *Agave* biomass have been found by other researchers (Xiong et al. 2014). Other microorganisms such as yeasts generally produce higher ethanol than the present research from *Agave* biomass fermentation (Caceres-Farffn et al. 2008; Lopez-Alvarez et al. 2012; Murugan and Rajendran 2013).

There has been a considerable interest in the production of ethanol using biomass fermentation on a large scale. There is a focus towards high yield of ethanol with the use of lignocellulosic biomass with the high productivity to reduce the cost of production. During the hydrolysis process of biomass using enzymes like cellulase, purified enzymes with optimized conditions give better result for degrading cellulose and hemicellulose of the biomass (Prasad et al. 2009).

Currently, one of the major problems with the production of bioethanol from lignocellulosic biomass is the high production cost of the cellulolytic enzymes. Most of the commercially available cellulolytic enzymes are not efficient for simultaneous saccharification and fermentation process. The cellulolytic microorganisms possessing hydrolytic and fermentative abilities are more efficient for the bioethanol production from lignocellulosic biomass (Limayem and Ricke 2012). In this research the two *Bacillus* strains could produce cellulase efficiently by using *Agave* biomass and helped in the hydrolysis and fermentation of this lignocellulosic biomass to produce ethanol.

Further, these strains could also be used potentially for the production of bioethanol from other lignocellulosic biomass too.

3.5 Morphology observation of *Agave* fiber

The control (untreated) *Agave* leaf surface was smooth after 7 days of incubation which indicates no degradation of the fibers (Fig. 7A & 7A'). The bacteria treated images show the broken cell wall with rough surface (7B & 7C). After the bacterial treatment, the fiber surface was broken forming a large number of crevasses. The degraded cell wall allows more cell wall degrading enzymes. Initially, the fine fibers are interwoven into a complex structure.

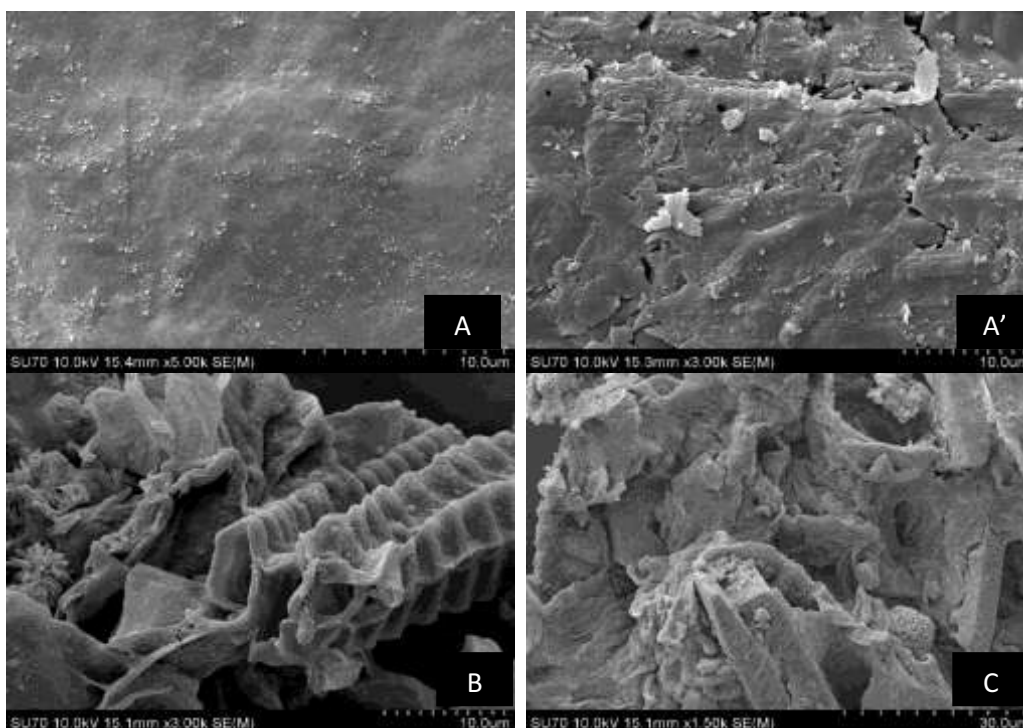


Figure 7 Scanning electron microscopy (SEM) images of agave fibers. (A& A') Untreated agave leaf structure (control) during an incubation period of 7 days (B & C) *Bacillus* sp. K1 and *Bacillus* sp. A0 treated agave leaf structures after 7 days of incubation.

The damage of cytoderm of the fiber helps to depolymerisation of cellulose and hemicellulose of *Agave*. Both of the *Bacillus* strains were able to damage the cytoderm of *Agave* fiber which helped to increase the cellulose and hemicellulose depolymerisation. This finding was similar with the findings of other researchers who

reported that *Bacillus* species have ability to degrade the cellulose, hemicellulose and lignin (Maki et al. 2012).

4. Conclusions

In this research, the two *Bacillus* species isolated from rotting wood samples were assayed for cellulase activity. Both of the strains exhibited the higher activity than many other bacterial and fungal species. While using *Agave* biomass as a source of carbon, the CMCase activity of K1 was 3.82 ± 0.24 U/ml and A0 showed this activity as 3.5 ± 0.12 U/ml. Based on SDS-PAGE analysis, the molecular weights of the cellulases produced by K1 and A0 were found ~ 36 kDa and ~ 40 kDa respectively. By using untreated *Agave* biomass, the maximum ethanol production was 0.435 g/g by strain K1. Similarly, isolate A0 produced 0.397 g/g ethanol on the 4th day of incubation. Both of these strains are of particular interest for producing maximum cellulase which might be valuable for biorefining industries for the production of bioethanol. These bacterial strains were able to change the morphology of *Agave* fiber. Also, the *Agave* biomass was found a good source of biomass during fermentation for bioethanol. Further research is required for improving the ethanol yield by using different fermentation conditions and for detecting the other important chemicals produced during the fermentation of *Agave* biomass.

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CHAPTER IV

Characterization of pectin depolymerising exo polygalacturonase by *Bacillus* sp. HD2 isolated from the gut of *Apis mellifera* L.

(Published- <http://dx.doi.org/10.7243/2052-6180-3-2>)

Abstract

Polygalacturonase is an important pectin degrading enzyme. The western honey bee (*Apis mellifera* L.) collects pollens from different flowers which are rich sources of pectin. The microbiota in the gut of the honey bee release polygalacturonase enzymes and help in pectin digestion. This study reports the isolation and characterisation of novel polygalacturonase producing *Bacillus* sp HD2 from honey bee's gut. This bacterial strain showed the maximum growth and enzyme production at 72 h of incubation. The exo polygalacturonase produced by this strain of *Bacillus* was optimal at 40 °C and exhibited the enzyme activity in a wide range of pH from pH 5-12. The polygalacturonase production was enhanced by using yeast extract (3%) in the production medium and the enzyme activity was stimulated by Ca²⁺ (2 mM) and SDS (200 mM). Biomass of apple's peel (1%) was found as an excellent source of carbon for the polygalacturonase production in fermentation medium (17.11 ± 0.46 μMml⁻¹min⁻¹). The SDS-PAGE analysis confirmed two bands of protein with polygalacturonase activity at ~36 kDa and ~72 kDa. Based on its properties, this enzyme has potential application for animal feedstock, degumming of ramie and fruit juice-processing.

Key words: Pectin, polygalacturonase, isolation, *Bacillus*, biomass

1. Introduction

The natural diet of honey bee is mainly plant nectar and pollen. Pectin is an important polysaccharide which helps to form different layers in the pollen wall (Aouali et al. 2001). The honey bee mid gut bacteria help to facilitate the digestion of pollen by releasing pectin degrading enzymes (Klungness and Peng 1984). Engel et al. (2012) identified the genes which encode pectin degrading enzymes in bacteria of honey bee's gut. Pectin digestion in the honey bee gut might be helpful in resulting the release of nutrients from pollen. Also, pectin has been shown to be toxic to honey bees (Barker 1977) and its digestion by gut bacteria might help the bees avoiding intoxication. Pectin hydrolysing enzymes are known as pectinolytic enzymes or pectinases. There are three types of pectinases; pectin methyl esterase, pectin lyase and polygalacturonase. Pectin methyl esterase helps in the de-esterification of pectin by breaking ester bond between the methyl group and carboxylic acid of galacturonic residues. Pectin lyase breaks the glycosidic bonds between galacturonic residues by trans-elimination reaction and polygalacturonase (PG) helps in the hydrolysis of α -1, 4 glycosidic bonds of pectin polymer converting into its galacturonic acid units (Contreras Esquivel and Voget 2004). Polygalacturonases have industrial importance since they help to decrease the viscosity, increase the fruit juice yield and help to study the crystalline structure of fibers (Souza et al. 2003).

In the industrial sector, acidic pectinases are used in the extraction and clarification of fruit juices (Rombouts and Pilnik 1986), whereas, alkaline pectinases have great commercial importance in the treatment of effluents discharged from fruit processing units (Tanabe et al. 1987), coffee and tea fermentation, oil extraction, processing and degumming of plant fibres such as ramie (Baracat et al. 1989; Kashyap et al. 2001). The alkaline pectinases have also been used in several biotechnological

processes, like purification of plant viruses (Salazar and Jayasinghe 1999) and paper making (Reid and Ricard 2000). The major source of acidic pectinases are fungi. The alkaline pectinases are produced from bacteria, mainly *Bacillus* spp. Although many fungi produce polygalacturonases (Birgisson et al. 2003), they are slow growing. The aim of this study was to isolate the bacterial strain capable of producing polygalacturonase from the gut of western honey bee (*Apis mellifera* L.) and optimization for maximum polygalacturonase production by the isolated strain.

2. Material and methods

2.1 Growth media

For the bacterial growth, the media used include pectin agar (pectin 5g l⁻¹, NaNO₃ 1 g l⁻¹, K₂HPO₄ 1 g l⁻¹, KCl 1 g l⁻¹, MgSO₄ 0.5 g l⁻¹, yeast extract 0.5 g l⁻¹, agar 15 g l⁻¹ and LB (Luria-Bertani) liquid media (10.0 g l⁻¹ peptone, 5.0 g l⁻¹ yeast extract and 5.0 g l⁻¹ NaCl).

2.2 Isolation of bacteria using pectin agar medium

The bacterial strains were isolated from the gut of western honey bee (*Apis mellifera* L.). For the isolation of bacterial strains, the gut sample from a honey bee was suspended in 10 ml of sterile potassium phosphate buffer solution (PBS). After vortexing the solution, a 10X serial dilution of the suspension was made in 1X PBS (pH 7). Thereafter, 100 µl of each dilution in the series was spread onto the surface of pectin agar using the standard spread plate technique. The plates were incubated at 28 °C for 24 h. The bacterial colonies were selected based on their morphology (size and color) (Holt et al. 1994). Pure cultures were repeatedly sub cultured on pectin agar plates and maintained for enzyme studies.

2.3 Screening of Isolates for polygalacturonase activity

The isolates were screened for polygalacturonase activity by culturing it in the pectin agar medium. The clear zone around the colony was detected while testing it with potassium–iodide solution (Salomao et al. 1996). The strain showing maximum zone of hydrolysis was selected for further assay.

2.4 DNA isolation and 16S rDNA amplification

The polygalacturonase producing isolate HD2 was grown in LB broth for 24 h at 28 °C. Genomic DNA was isolated using Geneaid DNA extraction kit (FroggaBio, Canada) by following the manufacturer’s protocol. The DNA was amplified by using HAD-1(5’-GACTCCTACGGGAGGCAGCAGT-3’) and E1115R (5’-AGGGTTGCGCTCGTTGCGGG-3’) primers. The PCR reaction mixture contained Taq buffer (10X), MgCl₂ (25 mM), dNTPs (0.4 mM), primers (10 mM), Taq DNA polymerase (0.25 U/μl), ddH₂O (7.5 μl) and DNA template (3 μl). The PCR program used was as follows: primary denaturation 3 minutes at 95 °C, followed by 35 amplification cycles consisting of denaturing at 95 °C for 1 minute, annealing for 1 minute at 63 °C, and extension at 72 °C for 1 minute. Upon completion of 35 amplification cycles, a final extension step was done at 72°C for 10 minutes. The amplified DNA was visualized on 1% agarose gel to confirm size, quantity and purity. The PCR product was purified by using Geneaid PCR/Gel purification kit (FroggaBio, Canada). Then the purified PCR products were sent for sequencing to Eurofins Genomics (U.S).

2.5 Isolate identification

BLAST (Basic Local Alignment Search Tool) program of NCBI database (<http://blast.ncbi.nlm.nih.gov/>) was used for identifying the possible genus of the isolate from the sequencing result.

2.6 Polygalacturonase production media

The polygalacturonase production was assayed using submerged fermentation technique in Dubois salt medium (NaNO_3 0.1 g l^{-1} , K_2HPO_4 0.1 g l^{-1} , KCl 0.1 g l^{-1} , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 g l^{-1}) containing 1% pectin (Acros Organics, Practical Grade, Fisher Scientific, Canada). For the biomass fermentation, 1% biomass from different fruits/vegetables used as a source of carbon in Dubois salt medium.

2.7 Enzyme Assay and total protein determination

The isolate HD2 was further screened for the quantitative polygalacturonase activity and total protein estimation by transferring 7 μl of an overnight culture to 7 mL of Dubois pectin media (pH 7.0) in a glass culture tube. The cultures were incubated for up to five days. The enzyme in the culture medium was harvested in the 1st, 2nd, 3rd, 4th and 5th days of incubation. The enzyme activity was determined by measuring the release of reducing groups using the dinitrosalicylic acid reagent DNS assay (Miller 1959) using pectin as substrate for polygalacturonase activity. For this, 10 μl of enzyme supernatant was added to 90 μl of substrate buffer (0.5% pectin in 0.05 M potassium phosphate buffer, pH 6-13) and incubated at 40-50 °C for 15 min. The reducing sugar released as galacturonic acid was determined. The cell free supernatant was used to evaluate the total protein by Bradford assay (Bradford 1976) using bovine serum album as standard.

2.8 Optimization of polygalacturonase production

2.8.1 Effect of incubation time on polygalacturonase production

Effect of incubation time on polygalacturonase was studied by incubating the bacteria in production medium for different time intervals (24 h, 48 h, 72 h, 96 h, and 120 h). Also, the growth of bacteria was monitored by measuring the optical density (O. D) at 600 nm and the cell free supernatant was used for the enzyme assay.

2.8.2 Effect of pH and temperature on polygalacturonase activity

The impact of the pH on enzyme activity was determined by performing the assay at different pH levels from pH 5.0 to 13.0 with cell free supernatant. For this, different pH buffers were used (Citrate buffer pH 5-6, PBS buffer pH 7-8, Glycine + NaOH buffer pH 9-11 and KCl + NaOH buffer pH 12-13). Similarly, the polygalacturonase activity was assessed at different temperatures ranging from 30 °C to 70 °C.

2.8.3 Effect of different nitrogen source on polygalacturonase Production

In order to study the impact of nitrogen source on enzyme production, bacterial culture was grown in a fermentation medium containing various nitrogen sources (0.5% w/v). The different nitrogen sources were yeast extract, peptone, urea and ammonium sulphate. Similarly, the effect of concentration of yeast extract on polygalacturonase production by bacterial strain HD2 was studied by using different concentrations of yeast extract ranging from 1 to 5 % (w/v) in the production medium.

2.8.4 Effect of metal ions on polygalacturonase activity

The effect of different metal ions, Ca^{2+} , Co^{2+} , Mg^{2+} and Zn^{2+} in their chloride and sulphate salts, on the activity of polygalacturonase was determined by performing the enzyme assay in the presence of these metal ions (2 mM) at 40 °C for

15 min. For this assay, the reaction mixture contained 10 µl enzyme supernatant, 10 µl metal ion, 30 µl buffer and 50 µl 1% substrate (pectin). Further, the effect of different concentration of Ca²⁺ from 1 mM to 5 mM, was determined by performing the polygalacturonase assay at 40 °C for 15 min.

2.8.5 Effect of detergents on polygalacturonase activity

The effects of detergents Sodium Dodecyl Sulphate (SDS, 10 mM) and Triton X-100 (10%) were observed on the polygalacturonase activity. The assay conditions were same as that of metal ions except the detergents were used instead of metal ions. Also, different concentrations of SDS were used ranging from 50 mM to 300 mM to determine the effects of these concentrations on polygalacturonase activity.

2.8.6 Effect of biomass on polygalacturonase production

For the assay of polygalacturonase production in fermentation state, different fruit peels (apple, pomengrate and orange) and vegetables peels (potato and squash) were used. The fruit/vegetables' peels were dried and powdered by a grinding machine. The overnight LB broth grown bacterial strain was inoculated in a powdery biomass (1% w/v) with Dubois salt medium for the fermentation and the polygalacturonase activity was determined at 72 h of incubation.

2.9 SDS-Polyacrylamide gel electrophoresis (PAGE)

The enzyme was confirmed by using SDS- PAGE. Ten percent SDS-PAGE was performed on the polygalacturonase by the method described by Laemmli (1970) using Bio-Rad electrophoresis apparatus. The protein marker and enzyme were allowed to run simultaneously to determine the molecular weight of the enzyme. After completion of the electrophoresis, Coomassie Brilliant Blue R-250 was used to stain the gel. The gel containing 0.25% pectin was used for detection of PG activity. The gel was then

washed with 2% Triton X-100 for 30 min, it was transferred in pH 11 and then incubated at 40 ° C for 30 min. After that the gel was stained with 0.1% Congo Red solution and the stained was removed with 1M NaCl to visualize the clear bands of PG activity.

2.10 Statistical analysis

All the experiments were performed in triplicate and the results are expressed in terms of mean \pm SD (standard deviation). The statistical analysis of data was performed by one way Analysis of Variance (ANOVA) followed by Duncan's multiple comparison test and Tukey's HSD test ($p < 0.05$) using SPSS system. Tests for normality by Kolmogorov-Smirnov test and Shapiro-Wilk test were performed to check if assumptions of ANOVA were met before the analysis.

3. Results and Discussion

In this study, four bacterial strains were isolated from the gut of western honey bee (*Apis mellifera* L.). For screening purposes, these bacteria were grown in pectin agar plate and pectinolytic activity was detected using plate assay. The isolate HD2 showed maximum pectinolytic activity on pectin agar plate. Sequencing results were successfully obtained for this isolate's 16S rDNA PCR products. The DNA sequences were analysed by the nucleotide BLAST feature of the NCBI database to obtain possible identities based on homology. From the BLAST, the isolate was 99% similar to genus *Bacillus*. The sequence of this strain was successfully uploaded to NCBI Genbank database (Accession no. KP676929). This strain was further screened for exo polygalacturonase activity.

3.1 Bacterial Growth, polygalacturonase production and the total protein determination

Bacterial growth is an important factor for the production of the enzymes. The growth factors are also of prime importance in industrial production for high production of enzymes for different applications. Bacteria show high levels of pectinases in pectin supplemented media (Soriano et al. 2005). The pattern of polygalacturonase production with reference to incubation period was monitored and the results showed that the *Bacillus* sp. HD2 showed considerable growth and maximum enzyme production ($12.44 \pm 0.8 \mu\text{molml}^{-1}\text{min}^{-1}$) at 72 h of incubation (Fig. 1) which was higher than other strains of *Bacillus* like *B. sphaericus* MTCC 7542 (Jayani et al. 2010). The production of polygalacturonase was decreased gradually after 72 h of incubation. Also, the total protein in the supernatant was found maximum ($118.22 \pm 5.41 \mu\text{g/ml}$) at 72 h of incubation (Fig. 2) which was significantly different to that of the PG activity at 24 h, 96 h and 120 h. The decrease in the growth, supernatant protein and enzyme activity after 72 h of incubation might be due to the loss of bacterial vitality which occurs after the limited growth.

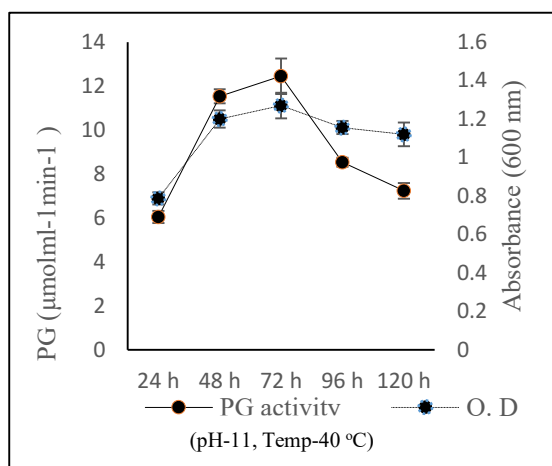


Figure 1 Absorbance and activity of PG from (Polygalacturonase) from *Bacillus* sp. HD2

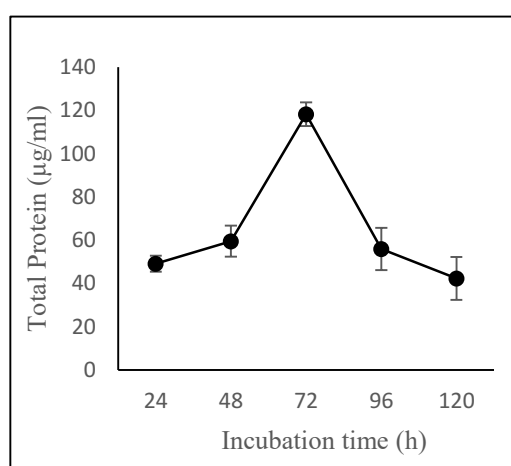


Figure 2 Total protein in the supernatant *Bacillus* sp. HD2 at different time

3.2 Effect of pH and temperature on enzyme activity

pH plays a significant role in the stability of enzyme activity. The present polygalacturonase from *Bacillus* strain was stable in the broad range of pH. The maximum polygalacturonase activity was found at pH 11 which was significantly different to the PG activity at pH 5, pH 6, pH 7, pH 8, pH 12 and pH 13 ($p < 0.05$). The relative enzyme activity showed that the PG was also active even in acidic pH retaining its activity of 56% at pH 5 (Fig.3A). Similar results of different alkaline polygalacturonase by different species of *Bacillus* were reported by other researchers (Anam and Zakia 2012; Dave and Vaughn 1971; Karbassi and Vaughn 1980; Nagel and Vaughn 1961). Kapoor et al. (2000) also reported that the polygalacturonase produced by *Bacillus* sp. MG-cp-2 was stable in alkaline conditions pH 7-12. Similarly, a polygalacturonase from *Klebsiella* sp. Y1 was reported to be stable in a wide range of pH (2-12) in digestive tract of sheep (Yuan et al. 2012).

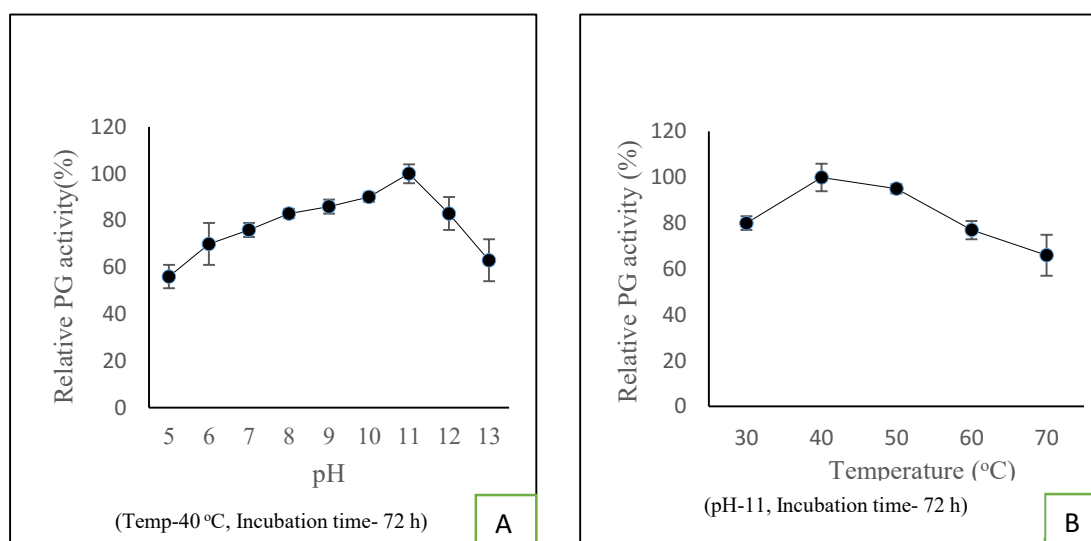


Figure 3 Effect of pH (A) and temperature (B) on the relative activity of PG (Polygalacturonase) from *Bacillus* sp. HD2

Like pH, temperature stability of enzyme is important for industrial application (Bhatti et al. 2006). The effect of temperature on polygalacturonase production by strain HD2 was studied at different temperature ranging from 30 °C to 70 °C. The enzyme

was stable in a wide range of temperature and an increased polygalacturonase activity was found at 40 °C which was significantly different to the PG activity at 30 °C, 60 °C and 70 °C ($p<0.05$). The PG was stable even at 70 °C retaining 66% of its enzyme activity (Fig. 3B). A moderate temperature is important for longer incubation period to reduce the cost of enzyme production (Anam and Zakia 2012). The results were similar with the temperature optimization for polygalacturonase from *Bacillus subtilis* DT7 (Kashyap et al. 2000).

3.3 Effect of different nitrogen source (0.5%) on PG activity

Maximum polygalacturonase production was achieved when yeast extract (YE) was used in culture medium (Fig. 4A). This activity was significantly different to the PG activity when urea and ammonium sulphate were used as nitrogen sources ($p<0.05$). It might be because of the fact that yeast extract has essential vitamins, minerals and amino acids which are helpful for bacterial growth and enzyme production. Similar reports were found in *Bacillus* sp. by other researchers (Rehman et al. 2012). Yeast extract is helpful for exo pectinase expression (Aguilar et al. 1991; Rehman et al. 2012). Different concentrations of yeast extract were used for enzyme production and it was found that maximum enzyme production was achieved when 3% yeast extract was used in the medium. On increasing the concentration of YE over 3%, the PG relative activity was decreased gradually (Fig. 4B).

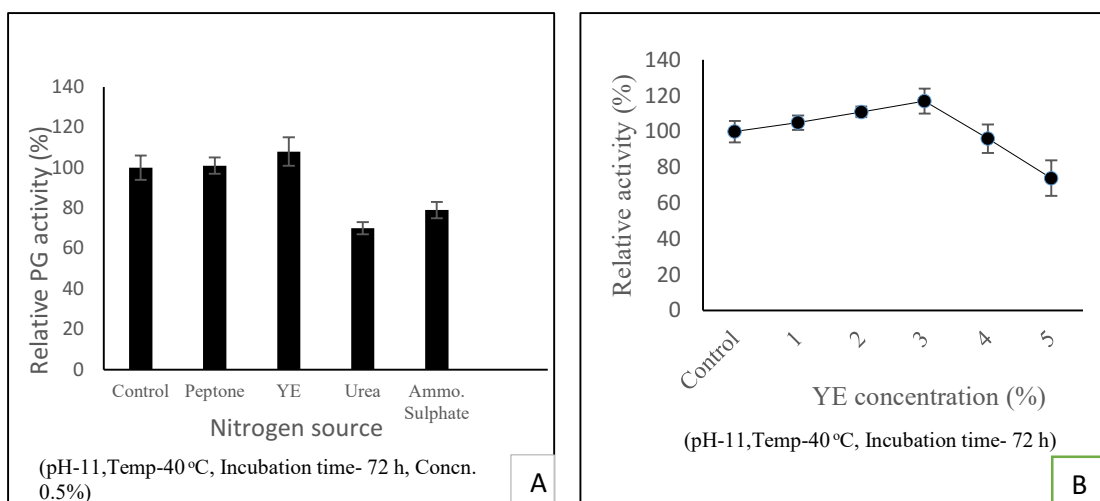


Figure 4 Effect of nitrogen source (A) and YE (B) on the relative activity of PG (Polygalacturonase) from *Bacillus* sp. HD2 (YE- Yeast extract, Ammo. Sulphate- ammonium sulphate)

3.4 Effect of metal ions on PG activity

The activity of polygalacturonase was enhanced by Ca^{2+} over Co^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} . All of these metal ions had no remarkable inhibition effects on PG activity (Fig. 5A). While using different concentrations of Ca^{2+} , maximum polygalacturonase was produced at 2 mM of Ca^{2+} . Further, the PG activity was decreased with the higher concentrations of Ca^{2+} (Fig. 5B). Similar results of polygalacturonase stimulation by Ca^{2+} were found in *Bacillus* sp. and *Klebsiella* sp. Y1 (Des Raj et al. 2003; Kobayashi et al. 2001; Yuan et al. 2012). The metal ions Mg^{2+} , Ca^{2+} , Zn^{2+} , Co^{2+} and Mn^{2+} have been found to stimulate the exopectinase activity by *Bacillus* GK-8 (Dosanjh and Hoondal 1996).

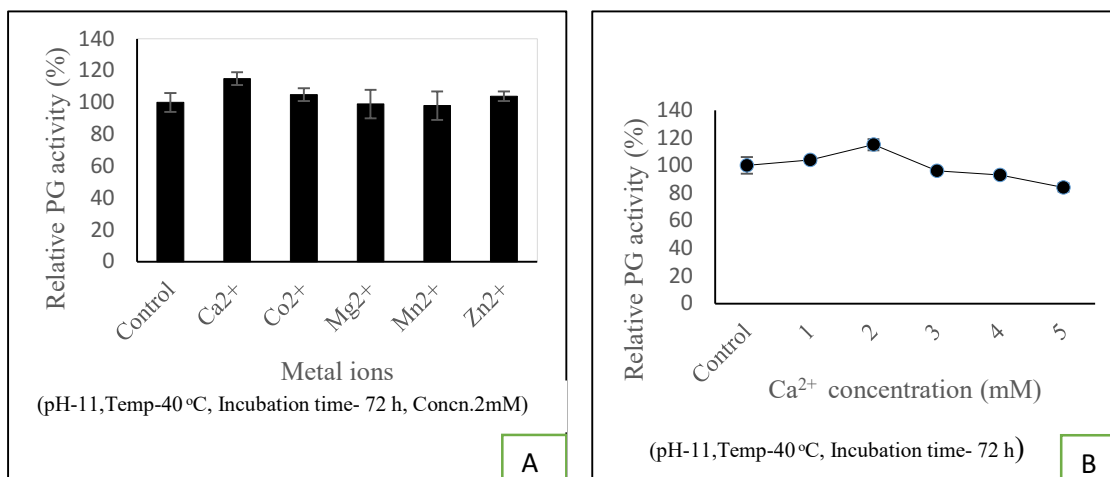


Figure 5 Effect of metal ions (A) and Ca²⁺ (B) on relative activity of PG (Polygalacturonase) from *Bacillus* sp. HD2

3.5 Effect of detergents on PG activity

The polygalacturonase was tolerant to surfactant SDS (Fig. 6A) and was reduced by Triton X-100. The activity in SDS was significantly different ($p < 0.05$) to that of the activity in Triton X-100. The stimulation of PG activity by SDS might be due to the increase affinity of active site of the enzyme to the substrate by lowering the surface tension by this detergent. The polygalacturonase activity was found to be most active when 200 mM of SDS was used (Fig 6B). The activity of PG from *Bacillus* sp. was stimulated by SDS as reported by other researchers (Kapoor et al. 2000), while Kobayashi et al. (2001) reported 10% reduction in polygalacturonase from *Bacillus* strain by SDS. In other reports, the polygalacturonase from *Sporotrichum thermophile* was slightly activated by Triton X-100 but the enzyme activity was completely lost by SDS (Kaur et al. 2004). The tolerance of SDS by PG might be helpful in the industries which require surfactant tolerant reactions in alkaline conditions.

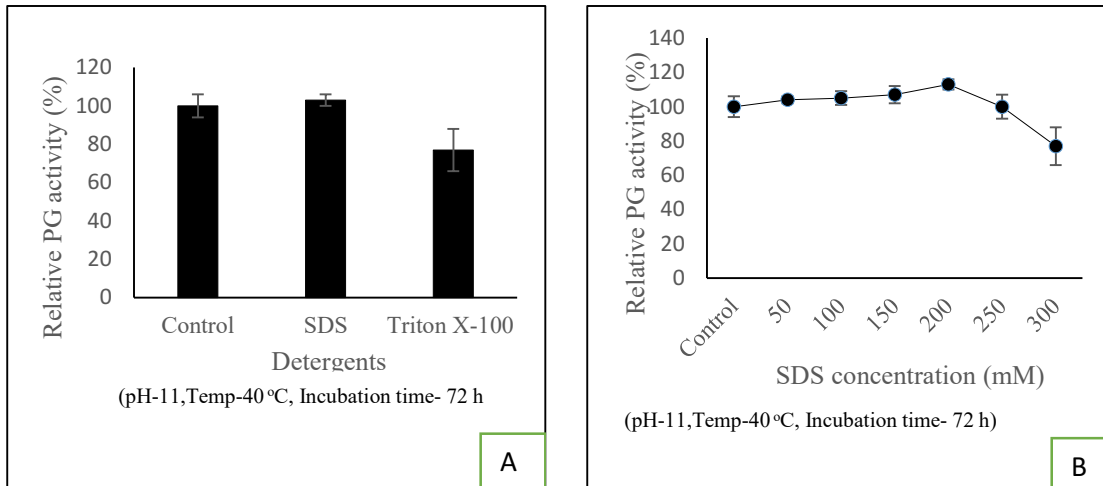


Fig. 6 Effect of detergents (A) and SDS concentration (B) on relative activity of PG (Polygalacturonase) from *Bacillus* sp. HD2

3.6 Effect of different biomass in PG production during fermentation

When the enzyme production profile of *Bacillus* sp. was studied by using different fruit and vegetable peels, the enzyme was assayed at 72 h of incubation. The biomass of apple peel (1%) was found significantly different to other biomass ($p < 0.05$) for the production of polygalacturonase ($17.11 \pm 0.46 \mu\text{molml}^{-1}\text{min}^{-1}$) during fermentation (Fig. 7).

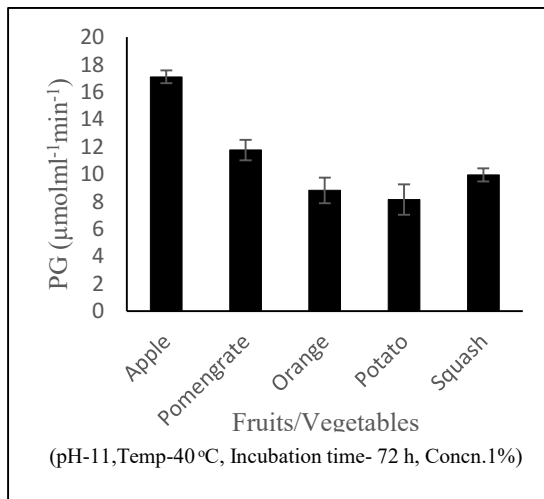


Figure 7 Effect fruits/vegetables biomass as carbon source on the production of PG (Polygalacturonase) from *Bacillus* sp. HD2

Apple biomass is an excellent source of polygalacturonase and other pectolytic enzymes (Berovic and Ostrovesnik 1997; Hours et al. 1988). However, Embaby et al.

(2014) reported that orange peel is an effective inducer (carbon source) for alkaline polygalacturonase by *Bacillus licheniformis* SHG10. Pectinases could be used to hydrolyse the pectin in pectin-rich agro-industrial wastes. The bacterial strain in its optimized conditions could be used to increase the polygalacturonase production at industrial scale.

3.7 SDS-PAGE analysis

The SDS-PAGE analysis of crude PG showed different protein bands. Out of which, two clear bands were found with positive enzyme activity with molecular weights of ~36 kDa and ~72 kDa (Fig. 8) indicating the possible presence of two main fractions of PGs. These bands could indicate the presence of different proteins or impurities.

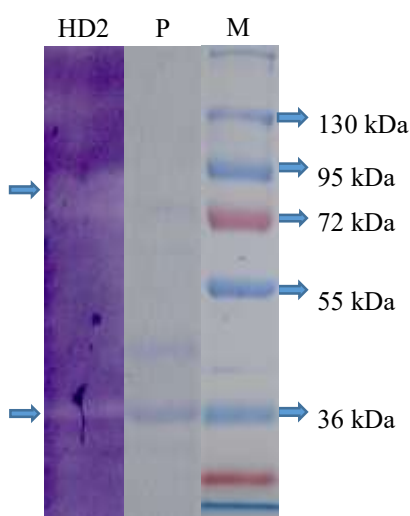


Fig. 8- SDS-PAGE of crude PG (Polygalacturonase) from *Bacillus* sp. HD2 (HD2-PG, P-Supernatant Protein, M-Marker protein; The molecular wt. of PG enzyme was found ~36 kDa and ~72 kDa)

This study was supported the findings of several researchers who reported the PGs with different molecular weights in different fungal and bacterial strains such as 38 and 61 kDa; 38 and 65 kDa; 63 and 79 kDa from *A. niger*, *A. japonicas* and *Penicillium frequentans*, respectively (Jayani et al. 2005). Similarly, molecular weights

of 36, 53 and 68 kDa; 66 kDa and 153 kDa were reported from *A. sojiae*, *Bacillus* sp. MBRL576 and *Bacillus licheniformis* KIBGE-IB21 respectively (Bhardwaj and Garg 2012; Rehman et al. 2015; Tari et al. 2008). Yuan et al. (2014) also reported the molecular weight of PG from *Klebsiella* sp.Y1 to be 72 kDa.

4. Conclusions

In this research, a new bacterial strain producing polygalacturonase was isolated from the gut of western honey bee (*Apis mellifera* L.) and identified as *Bacillus* sp. HD2 after 16S rDNA sequence analysis. Maximum production of polygalacturonase by this strain was achieved at 40 °C after 72 h of incubation ($14.31 \pm 0.54 \mu\text{molml}^{-1}\text{min}^{-1}$). A novel, alkaline active and temperature stable polygalacturonase has been produced from this *Bacillus* sp HD2 showing molecular weights of ~36 kDa and ~72 kDa. The enzyme activity was enhanced by metal ions Ca^{2+} and was tolerant to detergent SDS. On the basis of these properties, the polygalacturonase from this bacterial strain qualifies for use in the depectinization of pectic wastewaters from industries, as alkalophilic pectinolytic microbes help in the easy removal of pectic material and render it easily decomposed by activated sludge treatment. In addition to these properties, some additional features like enhanced production by yeast extract and apple biomass in the growth medium ($17.11 \pm 0.46 \mu\text{molml}^{-1}\text{min}^{-1}$) and the activity of enzyme in a wide range of pH, indicate the potential use of this organism at commercial level for animal feedstock, degumming of ramie and fruit juice-processing. Further, these results might be helpful to study the enzymatic pectin degradation mechanism in western honey bee (*Apis mellifera* L.).

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CHAPTER V

Conclusions and future directions

Microorganisms are important sources of industrially important enzymes like cellulases and pectinases. Bacteria offer several benefits over other microorganisms as their growth is fast and they are easy to culture even in small laboratory settings. Also, they have been widely used in the field of molecular biology, so they may easily be manipulated for gene transformation. The current study was focussed on the characterization of a novel cellulase and pectinase producing bacteria.

Seventeen cellulase producing bacterial strains were isolated from wood and the isolate K1 was further screened for its quantitative production of carboxymethyl cellulase. This bacterial isolate produced 5.21 ± 0.21 U/ml of cellulase which was higher than several other strains reported by other researchers (Gautam et al. 2011; Korpole et al. 2011; Meng et al. 2014; Yang et al. 2014). The working parameters of this cellulase were optimized and the maximum enzyme activity was found at pH 6, 50 °C and after 72 h of incubation. The production media containing 1% peptone and 1% lactose were found to increase the enzyme production significantly than other sources of nitrogen and carbon used in the experiment. Further, the isolates K1 and A0 fermented untreated *Agave* biomass, producing 0.435 g/g and 0.397 g/g of ethanol respectively. The SDS-PAGE analysis showed the molecular weights of the cellulases produced by K1 and A0 to be ~36 kDa and ~40 respectively. Based on the 16S rDNA sequencing analysis, the isolates K1 and A0 were identified and their sequences were successfully uploaded to NCBI gene bank data base as *Bacillus* sp. K1 (Accession no. KP987117) and *Bacillus* sp. A0 (Accession no. KP974676) respectively.

Similarly, a polygalacturonase (PG) producing bacterial strain was isolated from the gut of western honey bee (*Apis mellifera* L.). This isolate was identified as *Bacillus* sp. HD2 (Accession no. KP676929). The PG produced by this strain was optimum at pH 11, 40 °C and after 72 h of incubation ($14.31 \pm 0.54 \mu\text{molml}^{-1}\text{min}^{-1}$). This PG was active in a wide range of pH (pH 5-12) showing molecular weights of ~36 kDa and ~72 kDa. The enzyme activity was increased by Ca^{2+} and was tolerant to detergent SDS. Further, this *Bacillus* isolate could produce polygalacturonase using biomass of apple peels ($17.11 \pm 0.46 \mu\text{molml}^{-1}\text{min}^{-1}$). Based on its features, this enzyme is useful for several industrial applications and to understand the pectin digestion mechanism in the honey bee.

Future studies can be done in the identification and cloning of the genes like β -1, 4 endoglucanase in these cellulase producing isolates; which make the industrial utilizations of these *Bacillus* spp K1 and A0. The strain improvement for enhanced cellulase production can be achieved by using different techniques such as mutagenesis (Abdullah et al. 2014) and metabolic engineering (Lin et al. 2014) for the hyper production of multiple cellulolytic enzymes and effective bioconversion of lignocellulosic biomass to bioethanol and value added products. There are other important bioproducts produced by the fermentation of lignocellulosic biomass that can further be detected by using different analytical methods such as GC-MS, LC-MS and HPLC. Also, there exist some challenges like enzymatic hydrolysis of biomass due to the lack of efficient enzymes. So, it is important for reducing the enzyme production cost by developing the organisms which can help in fermentation efficiency and convert almost all the fermentable sugars into biofuels and bioproducts. Tremendous focus is required for the characterization, understanding and overcoming the barriers for enzymatic hydrolysis of different raw material which is essential for developing

economically competitive processes using enzymatic treatments. Similarly, improved fermentation technology and media optimization also help to improve efficiency of cellulases and the yield of end products.

Most of the organisms degrading the lignocellulosic biomass have end product inhibition reducing the rate of enzyme synthesis. This leads to the incomplete utilization of the biomass. Co-culture of different strains might be helpful to produce cellulase complexes in adequate quantity and helpful for the degradation of biomass efficiently (Maki et al. 2014). However, this has achieved limited success because of induced feedback inhibition among the species. To overcome this problem, development of mutant strains, genetic engineering such as modification of pathways and gene expression with desirable pathways may be helpful because this helps to enhance the bioethanol and organic acids.

Like cellulases, pectinases can also be applied to improve the quality and yield of final products in different industrial processes. So, it is important for the investigation of physico-chemical characteristics of new pectinases. In the future, research should be focussed more on the elucidation of the regulatory mechanism of enzyme secretion which will be helpful for the mechanism of action of these enzymes on different substrates. Cloning and expression of polygalacturonase producing genes always help to produce the enzymes for possible industrial applications (Chen et al. 2014). This will lead to valuable tool for producing the enzymes efficiently.

The processing and enzymatic hydrolysis of plant biomass has become the crux of future research. The enzymes such as cellulases and pectinases are being produced commercially for their wide applications in food, animal feed, fermentation, pulp and paper and textile applications. So, the future research should be focussed on the microbial production of these enzymes by using biotechnological tools for improving

the enzyme activities. Because the study of biochemical, regulatory and molecular aspects by using the molecular biology tools will be helpful in genetic engineering of cellulases and pectinases that are robust with respect to their pH and temperature tolerance. Additional physical parameters such as aeration and agitation should be optimized for the production of these enzymes and their interaction effects are required to be addressed. This may play a significant role on the use of these enzymes in industrial scale. Also, the enzymatic bioconversion of agro wastes and the identification of the end products will be helpful to keep the environment clean and production of value added products.

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