M. Tech (Industrial Biotechnology)

Khyati Joshi

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SCREENING, ISOLATION AND CHARACTERIZATION OF CELLULASE PRODUCING BACTERIA FROM ENVIRONMENT

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Submitted by:

Khyati Joshi
2K20/IBT/06

Under the supervision of

PROF. PRAVIR KUMAR
DR. RASHMI KATARIA

DEPARTMENT OF BIOTECHNOLOGY

DELHI TECHNOLOGICAL UNIVERSITY
(Formerly Delhi College of Engineering)
Bawana Road, Delhi-110042

MAY, 2022

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DELHI TECHNOLOGICAL UNIVERSITY
(Formerly Delhi College of Engineering)
Bawana Road, Delhi-110042

CERTIFICATE

We hereby certify that the project dissertation titled "Screening, isolation and characterization of cellulase producing bacteria from environment" which is submitted by **Khyati Joshi, 2K20/IBT/06**, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology, is a record of the project work carried out by the student under our supervision. To the best of our knowledge this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

Prof. Pravir Kumar
Supervisor
Head of Department
Department of Biotechnology
Delhi Technological University

Dr. Rashmi Kataria
Supervisor
Assistant Professor
Department of Biotechnology
Delhi Technological University

DELHI TECHNOLOGICAL UNIVERSITY
(Formerly Delhi College of Engineering)
Bawana Road, Delhi-110042

CANDIDATE'S DECLARATION

I, **Khyati Joshi, 2K20/IBT/06** student of M.Tech (Industrial Biotechnology), hereby declare that the project Dissertation titled "**Screening, isolation and characterization of cellulase producing bacteria from environment**" which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the requirement for the award of the degree of Master of Technology, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship or other similar title or recognition.

Place: Delhi
Date: May 30, 2022

Khyati Joshi
2K20/IBT/06

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Khyati Joshi
(2K20/IBT/06)

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ABSTRACT

Lignocellulosic biomass is the most plentiful matter on the earth. It holds potential to be transformed into value added chemicals like bioethanol, organic acid, bioplastics, enzymes, pigments etc. Lignocellulosic biomass is composed of cellulose, hemicellulose and lignin. Cellulose is the most prevalent organic matter on the planet and is a key glucose storage type. The fundamental step in the bioconversion of LCB into value-added bioproducts is based on the decomposition of cellulose by cellulase. Various groups of microbes including fungi and bacteria are known for their ability to produce cellulase. Although fungi have the most efficient cellulase activity, bacterial cellulase is gaining popularity because of its higher growth rate, better specific activity, stability and mass transfer. Therefore, identification of a novel and better bacterial strain with higher cellulase productivity and enhanced activity could be a breakthrough in the utilization of lignocellulosic biomass for various applications. In this study, cellulase producing bacteria were screened and isolated from soil sample. They were stained and qualitatively analysed for their cellulolytic activity using Congo-red staining. The bacteria showing maximum clear zone was used for further work. Its growth curve was analysed and its enzymatic activity was measured quantitatively using different assays. It was observed that maximum cell biomass was produced after 16 hours of incubation whereas maximum enzymatic activity of 0.4 IU/mL was observed after 14 hours of incubation.

Keywords: Cellulase; Lignocellulosic biomass; Congo-Red staining; Endoglucanase assay; Total reducing sugar assay

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LIST OF SYMBOLS AND ABBREVIATIONS

LCB- Lignocellulosic biomass

AFEX- Ammonia fiber/freeze explosion

ARP- Ammonia recycle percolation

PGPR- Plant growth-promoting rhizobacteria

CMC- carboxymethyl cellulose

DNS- 3,5-dinitrosalicylic acid

OD- Optical density

nm- Nanometer

µm- Micrometer

ºC- Degree Celsius

IU- International units

CHAPTER 1: INTRODUCTION

Lignocellulosic biomass (LCB) is one of the major plentiful organic materials in the biosphere and is a promising source of renewable energy. It is made up of three components: cellulose, hemicellulose and lignin, which confer it a recalcitrant structure [1]. Cellulose, which is the main component of LCB, is considered to be the best choice for replacing petroleum-based polymers because of its eco-friendly features such as renewability, biocompatibility and biodegradability [2]. Cellulose is the most prevalent matter on the planet, and cellulase is a common enzyme that breaks it down [3]. Microbes that degrade cellulose are extensively distributed in nature and have been isolated from a variety of habitats. Cellulases are used in a variety of industries, such as biofuel, food, feed, brewing, textile, laundry and agriculture industry [4]. For the cellulase action, it is necessary to for the cellulose to be accessible. To transcend the recalcitrant nature of LCB and exposing the cellulose, LCB must be pretreated. Pretreatment is used to change the physical characteristics and chemical composition/structure of lignocellulosic materials, making cellulose more accessible to enzymatic hydrolysis for sugar conversion [5]. There are several physical, chemical and biological methods of LCB pretreatment such as acidic, alkaline, microwave-assisted, steam explosion, organic solvent and enzymatic pretreatment. After the LCB has been pretreated, enzymatic hydrolysis can be carried out, in which cellulose is broken down into glucose units [6]. Enzymatic hydrolysis is a multistep reaction that takes place in a heterogeneous system, in which insoluble cellulose is initially broken down at the solid-liquid interface via the synergistic action of endoglucanase, exoglucanase and β-glucosidase [7]. Several microbes, most often bacteria and fungus, are known to produce cellulase [3]. Although fungi have the most effective cellulase activities, bacterial cellulase is gaining popularity due to their faster growth rate and larger potential for cellulase synthesis than fungi [8]. Apart from having a faster rate of bacterial growth, bacterial cellulases have a variety of benefits over fungal cellulases in terms of mass transfer, specific activity, and stability [9,10]. Therefore, the hunt for a new and superior bacterial strain with higher hyper cellulase productivity, increased activity, and high temperature, pH, and non-aseptic stability could make the procedure more economical [8].

CHAPTER 2: REVIEW OF LITERATURE

2.1 Lignocellulosic Biomass (LCB)

LCB such as agricultural waste represents the mainstream biomass present on the earth. LCB is a plentiful and renewable resource with significant potential in the production of second-generation biofuels and bio-based chemicals [11,12]. LCB is majorly comprised of cellulose, hemicellulose and lignin. These polymers form a hetero-matrix with variable relative composition based on the source and type of biomass (Table 1) [13].

Table 1. Composition of cellulose, hemicellulose and lignin in different LCB [4]

Lignocellulosic biomass	Cellulose	Hemicellulose	Lignin
Corn stalk	34.45	27.55	21.81
Rice straw	35.8	21.5	24.4
Corn stover	36.3	31.4	17.2
Sugarcane top	29.85	18.85	25.69
Bagasse	30	35	18
Sorghum bagasse	37	17.8	19.6
Sugarcane bagasse	44	27	24
Rapeseed	51.3	17.3	44

Typically, LCB consists of 9-80% cellulose, 10-50% hemicellulose and 5-35% lignin [14]. Cellulose is a polysaccharide that is comprised of a linear chain of D-glucose monomers united together by β-(1-4) glycosidic linkages. It is the most important structural component of green plants, algae, and oomycetes' major cell walls.

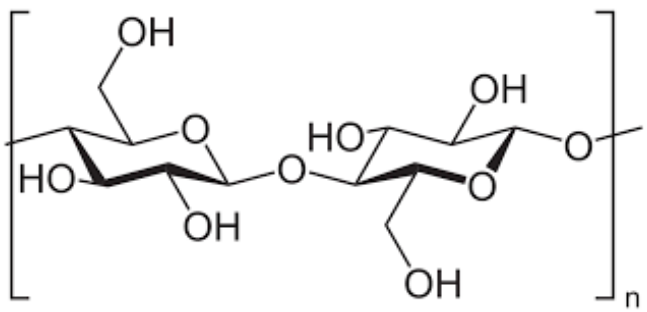


Fig. 2.1 Chemical structure of cellulose

Hemicellulose is a branched polymer with around 500 to 3000 sugar units. It is made up of various sugar units such as pentoses, hexoses and acetylated sugars. Hemicellulose strengthens the cell wall by cross-linking with cellulose or lignin.

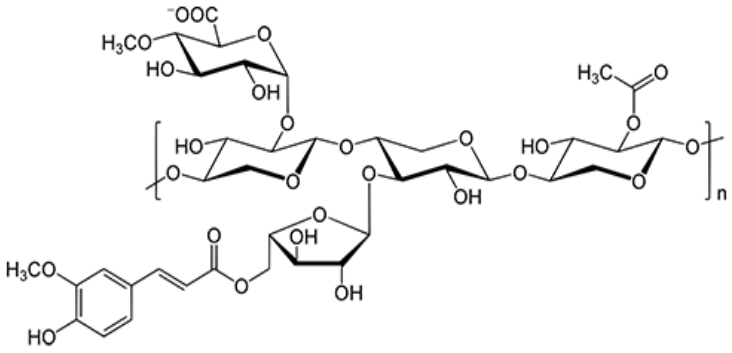


Fig. 2.2 Chemical structure of hemicellulose

Lignin is the 3rd major component of LCB that is accountable for providing structural integrity to vascular plants and algal tissues. It is a necessary constituent of plant walls, particularly in bark and wood. It is majorly amorphous in nature. It has rigidity and hardness due to cross-linked phenolic polymers in its structure. Lignin is a branched long chain polymer composed of three monomers: 4-propenyl phenol, 4-propenyl-2-methoxy phenol, and 4-propenyl-2.5-dimethoxyl phenol [15].

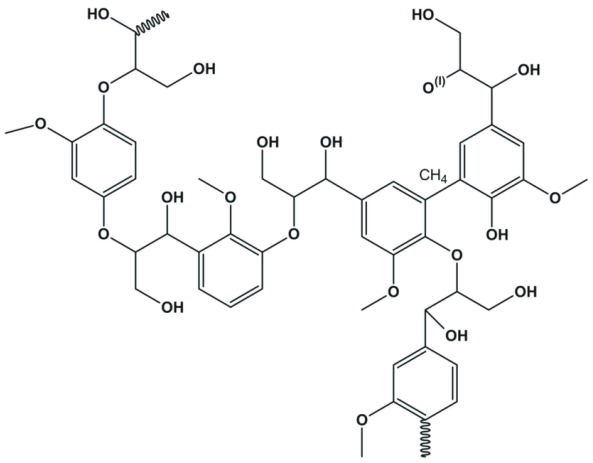


Fig. 2.3 Chemical structure of lignin

2.2 Pretreatment of Lignocellulosic Biomass

Pretreatment of LCB feedstocks is necessary for the structure modification and exposing of LCB fractions for better accessibility during enzymatic hydrolysis [16]. It is a necessary step to decrease the recalcitrant structure of LCB by removing lignin, disintegrating hemicellulose, decreasing cellulose crystallinity and improving the porosity of the LCB.

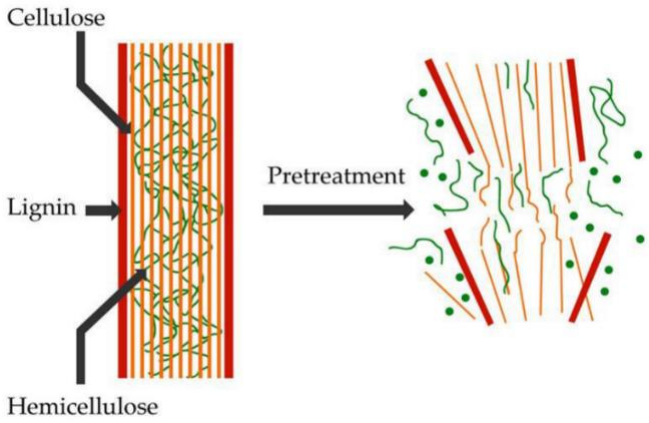


Fig 2.4 Pretreatment of LCB

Pretreatment is the most intensive phase in the entire biomass processing process. As a result, every effort should be made to reduce operating costs, improve effectiveness, and enhance the amount of lignocellulosic biomass that can be recovered [17]. Pretreatment methods can be divided into 4 different categories: Physical, chemical, physico-chemical and biological [18].

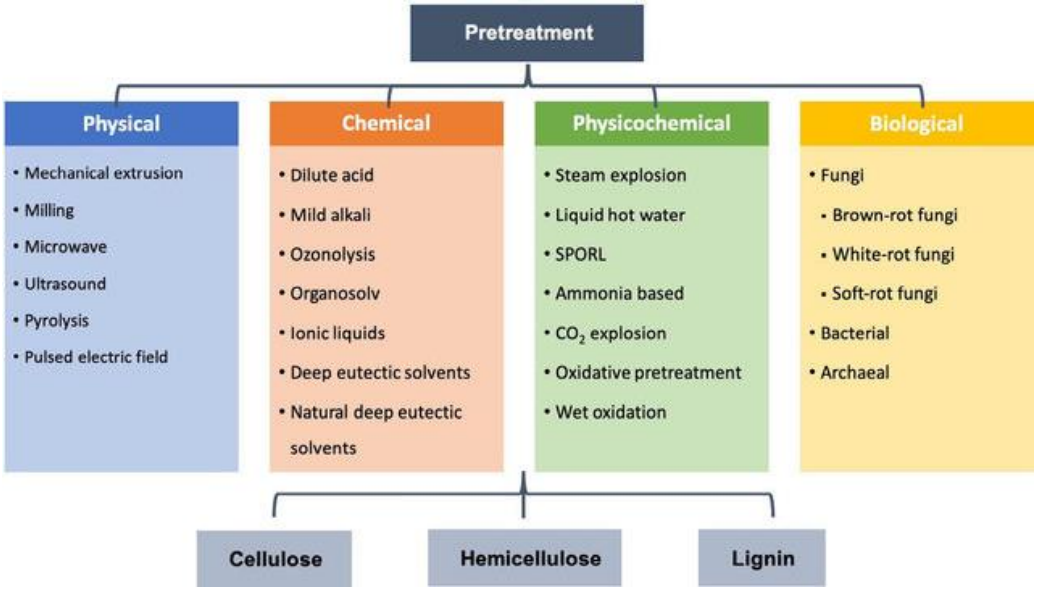


Fig 2.5 Different types of pretreatment methods

2.2.1 Physical pretreatment techniques

2.2.1.1 Milling

It is used to decrease the crystallinity of LCB. It can decrease the particle size to as small as 0.2 mm. Milling methods include ball, hammer, colloid, vibratory and wet disc milling, depending on the type of motorised equipment used. The type of milling equipment used, the milling period and the type of LCB used, all influence particle size and crystallinity reduction.

2.2.1.2 Microwave treatment

It is a non-traditional heating approach that is used to pre-treat LCB in the presence of an applied electromagnetic field. The dielectric polarisation in this approach promotes molecular collisions and generates heat energy, causing the complex lignocellulosic structure to be disrupted.

2.2.1.3 Ultrasonication

It is based on the concept of cavitation, which is achieved by using ultrasonic radiation. Cavitation creates shear pressures that break the LCB's complex network structure, allowing desirable components like cellulose, hemicellulose, and lignin to be extracted. The choice of solvents has been shown to be essential in optimizing ultrasonication pretreatment conditions.

2.2.1.4 Extrusion

It is one of the most prevalent preparation methods for LCBs. The foundation of this technology is the movement of 1 or 2 screws spinning into a compact barrel with temperature regulation. The raw materials are passed through the barrel at a high temperature (>300°C), where the lignocellulose's refractory structure is disrupted by the combined impacts of high temperature and shear pressures induced by the barrel's revolving screw blades [19].

2.2.2 Chemical pretreatment techniques

2.2.2.1 Alkali pretreatment

It comprises of the addition of alkali to LCB. This results in puffing up of biomass, causing increased internal surface and decreased crystallinity. This breaks the

connections between lignin and other polymers. Alkali pretreatment works best for LCB with low lignin content. As a result, the efficacy of this method is determined by the biomass's lignin level. The most commonly reported chemicals employed in alkaline pretreatment are NaOH, KOH, and Ca(OH)2. This method is based on using relatively mild process conditions for extended period of time.

2.2.2.2 Acid pretreatment

Pretreatment with acids such as H_2SO_4 improves the enzymatic hydrolysis of LCB to release sugars. The use of acid causes the van der Waals interaction, hydrogen and covalent bonding to break down, causing hemicellulose solubilization. Because glucomannan is more stable, the major process that happens is the breakdown of hemicellulose, particularly xylan. Dehydration of xylose galactose, mannose, and glucose can result in the formation of furfural and HMF under these conditions. Pretreatment with dilute acid hydrolysis, on the other hand, can lead to higher reaction rates and better cellulose lysis. In most situations, lignin is barely dissolved, but it is highly disturbed, resulting in increased cellulose susceptibility to enzymes [9].

2.2.3 Physico-chemical pretreatment methods

The common physico-chemical methods used for LCB pretreatment are steam explosion, ammonia fiber explosion (AFEX), supercritical fluid (SCF) pretreatment, ammonia recycle percolation (ARP) and liquid hot water (LHW) pretreatment [20].

2.2.3.1 Steam explosion

It is one of the extensively utilised pretreatment techniques because it combines chemical as well as physical procedures to destroy the lignocellulosic material's assembly. It uses hydrothermal approach to expose the LCB to high pressure and temperature for a brief term before immediately decompressing the system, breaking the fibril microstructure.

2.2.3.2 Ammonia fiber explosion

Another physicochemical process is the ammonia fiber/freeze explosion (AFEX), where LCB is exposed to liquid anhydrous NH_3 at increased pressure and moderate temperature before being rapidly decompressed. The mild temperatures are substantially lower than those used in the steam explosion process, resulting in lower energy consumption and lower overall expenses. Before being discharged to air

temperature, the NH_3 and LCB mixture is soaked for a length of time in a pressurised bioreactor. The speedy expansion of NH_3 causes swelling of the LCB feedstock, causing lignin-carbohydrate bond disruption, hemicellulose breakdown and fractional de-crystallization of cellulose, all of which result in an increased surface area for enzyme attack.

2.2.3.4 Ammonia recycle percolation

Many writers have combined ammonia recycle percolation (ARP) with AFEX procedure. Aqueous ammonia with a concentration of 5–15 percent (wt%) is passed via a loaded bed reactor holding the LCB feed at a rate of around 5 mL/min in this process. This technique has the benefit of AFEX in that it can remove the bulk of the lignin (75–85%) and solubilize majority of the hemicellulose (50–60%) while keeping a high cellulose content. This is owing to ammonia's selectiveness and its cability to ammonolyze lignin whilst solubilizing hemicellulose during prolonged holding durations.

2.2.3.5 Liquid hot water

To enhance the disruption of the LCB matrix, this method employs water at extremely high temperature and pressure. The cell penetration of the biomass by water, as well as the solubilisation of both hemicellulose and lignin by this liquid hot water acting as an acid, results in the synthesis of reactive cellulose fibres for the production of pentosans and the disruption of the entire lignocellulosic matrix [21].

2.2.4 Biological methods

Biological treatment using various varieties of rot fungi, is quickly being endorsed as a method for lignin exclusion from LCB. Microbes like brown-, white-, and soft-rot fungi are utilised in biological pretreatment methods to decompose lignin and hemicellulose in LCB. Brown rot fungi acts on cellulose primarily, while white and soft rot fungi targets cellulose as well as lignin. Lignin is degraded by the action of enzymes like peroxidase and laccase. Carbon and nitrogen supplies control the activity of these enzymes. For biological preparation of lignocellulosic materials, white-rot fungi are the most effective [22].

2.3 Enzymatic hydrolysis of lignocellulosic biomass

Bioconversion of LCB into fermentable sugars is necessary for industrially important product formation [7]. The feedstock for 90 percent of today's ethanol production is sugars and starch. However, cellulose and hemicellulose are the predominant sugars found in the environment. Enzymatic hydrolysis transforms the cellulosic portion of the biomass into sugars, which are then fermented into ethanol [23].

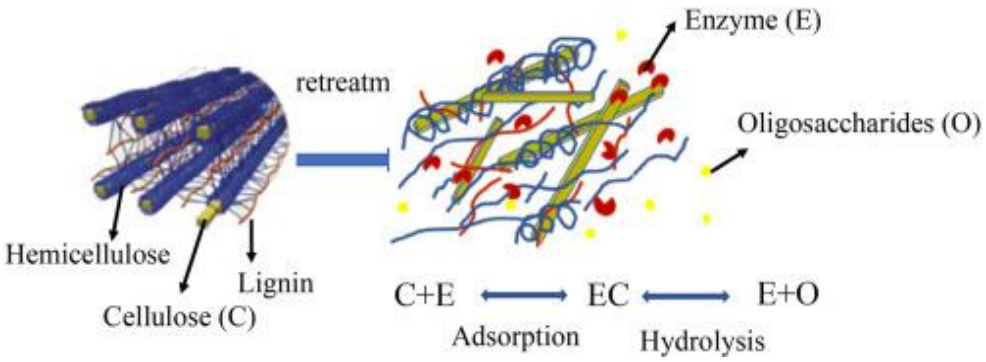


Fig 2.6 Enzymatic hydrolysis of LCB

At the molecular level, the complicated enzymatic hydrolysis of LCB involves the following steps:

2.3.1 The enzyme diffusion process

Diffusion is used to transport enzyme from liquid solution to the substrate. The intensity of hydrolysis is determined by the rate at which enzyme diffuses from liquid phase to LCB particles. Diffusion is a quick mechanism in general, and the early pace of hydrolysis is heavily impacted by it. Enzyme diffusion is dependent on several parameters such as the amount of enzyme used, fluid force, pore structure and temperature.

2.3.2 The enzyme adsorption process

In this step, enzyme diffuse farther into LCB via the pores present on its structure. Enzyme adsorption on LCB is a prerequisite for enzyme action, where cellulase acts on cellulose molecules. This step involves binding of cellulase to the active sites present on LCB particles in a step-wise manner.

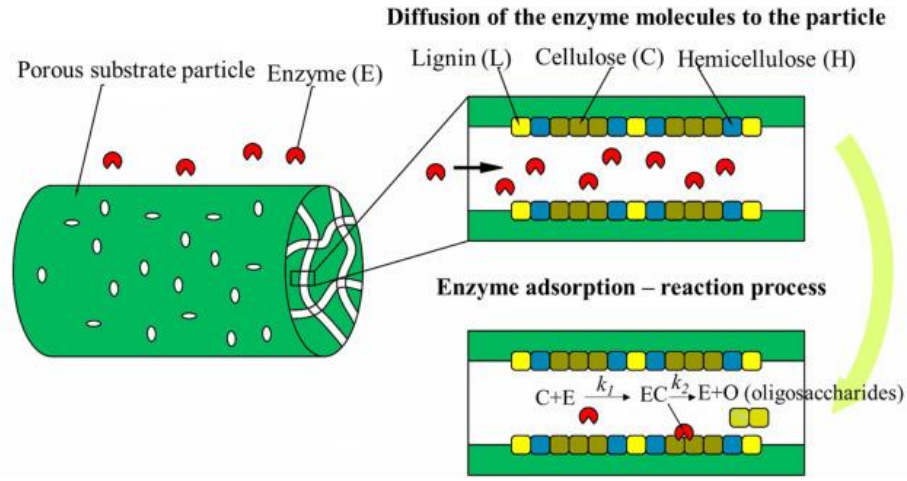


Fig 2.7 Mechanistic steps of enzymatic hydrolysis of LCB

2.3.3 The enzymatic hydrolysis process

On the cellulose surface, an enzymatic hydrolysis reaction occurs. Enzymatic hydrolysis is influenced by a variety of circumstances. The main factors influencing the reaction mechanism are: 1) operating conditions such as temperature, agitation, reaction time and solids loading; 2) enzyme properties such as activity, adsorption capacities and catalysis synergism and 3) substrate physical and chemical characteristics such as composition, surface area, crystallinity and pore size [24].

2.4 Cellulase and its mechanism of action

Cellulases are enzymes that catalyse the hydrolysis of the linear glucose polymer cellulose's – β-1, 4 bonds. They belong to the glucoside hydrolases family of enzymes [25]. Based on aa sequence similarity and crystalline structure, glycoside hydrolases, inclusive of cellulase, are divided into 115 families. Cellulase genes have now been cloned and described in vast numbers. They are classified into 13 families. In addition, more than 50 cellulases have 3D structures. They do, however, have a wide range of topologies [26].

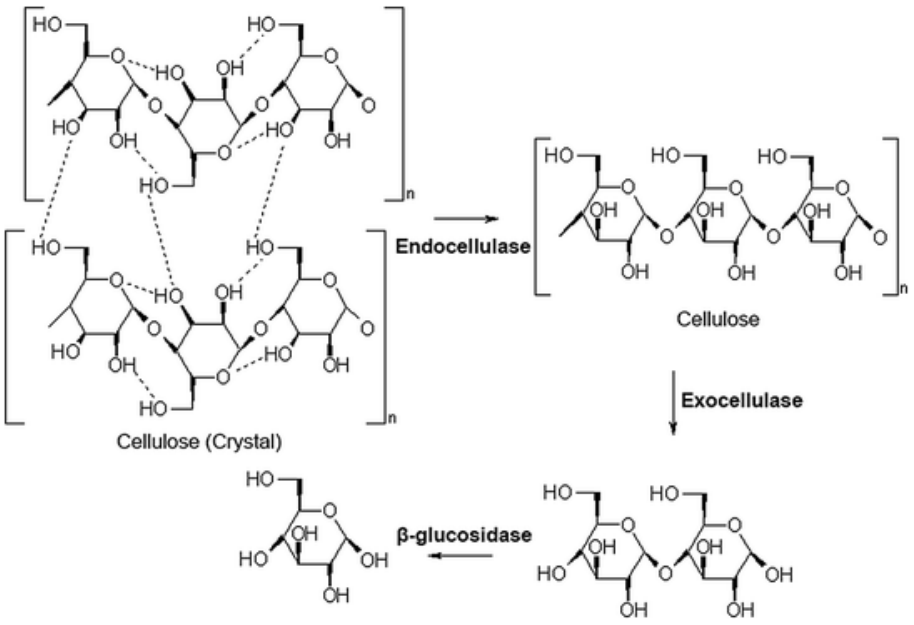


Fig 2.8 Mode of action of cellulase

Cellulases work by synergistic action of (i) endoglucanases, (ii) exoglucanases and (iii) β-glucosidases [25]. Endoglucanase preferably acts on cellulose that is amorphous. It builds new chain ends by haphazardly acting on internal bonds of weak amorphous regions of cellulose. Exoglucanase works by targeting the reducing or nonreducing extremities of cellulose chains to produce glucose units. Endoglucanase is different from exoglucanase in its ability to effectively act on crystalline cellulose materials like Avicel and cellooligosaccharides. Finally, from the nonreducing ends, β-glucosidase can hydrolyze cellobiose to glucose [25].

2.5 Cellulase producing microbes

Cellulases are a complex set of enzymes produced by various microbes such as fungus, bacteria and actinomycetes [27]. Typically, fungi produce extracellular cellulolytic enzymes whereas bacteria produce cellulolytic enzymes in a cell wall associated complex, called cellulosome [28].

Table 2. Major cellulase producing bacteria, fungi and actinomycetes

Major group	**Microorganism**	
	Genus	**Species**
Bacteria	*Bacillus*	*Bacillus sp*
	Pseudomonas	*P. cellulose*
	Clostridium	*C. acetobutylicum*
	Rhodothermus	*R. marinus*
Fungi	*Fusarium*	*F. solani*
	Trichoderma	*T. reesei*
		T. harzianum
	Aspergillus	*A. niger*
		A. nidulans
		A. oryzae
	Humicola	*H. insolens*
		H. grisea
	Penicillium	*P. brasilianum*
Actinomycetes	*Streptomyces*	*S. drozdowiczii*
		S. sp
		S. lividans
	Cellulomonas	*C. fimi*
		C.bioazotea
		C.uda
	Thermononospora	*T. fusca*
		T. curvata

2.6 Bacterial cellulase production

In bacteria, cellulase production is induced by nutritional content and process parameters such incubation time, temperature, pH and agitation speed. Cellulase production in bacteria may be significantly improved by optimising both nutritional and process parameters, and this can help with the development of industrial bioprocesses for enzyme synthesis [8]. Although fungus have the most efficient cellulase activities, there is growing interest in bacteria producing cellulase since bacteria have a faster growth rate in comparison to fungi and have a greater potential for cellulase synthesis. The hunt for a new and superior bacterial strain with higher hyper cellulase productivity, increased activity, and high temperature, pH, and non-aseptic stability could make the procedure more cost-effective [29]. Apart from, higher bacterial growth rate, bacterial cellulases have a number of advantages over fungal cellulases in terms of easier mass transfer, higher specific activity and greater stability [30,31]. Moreover, it is easier to carry out genetic recombination in bacteria in comparison to fungi [30].

2.7 Applications of cellulase

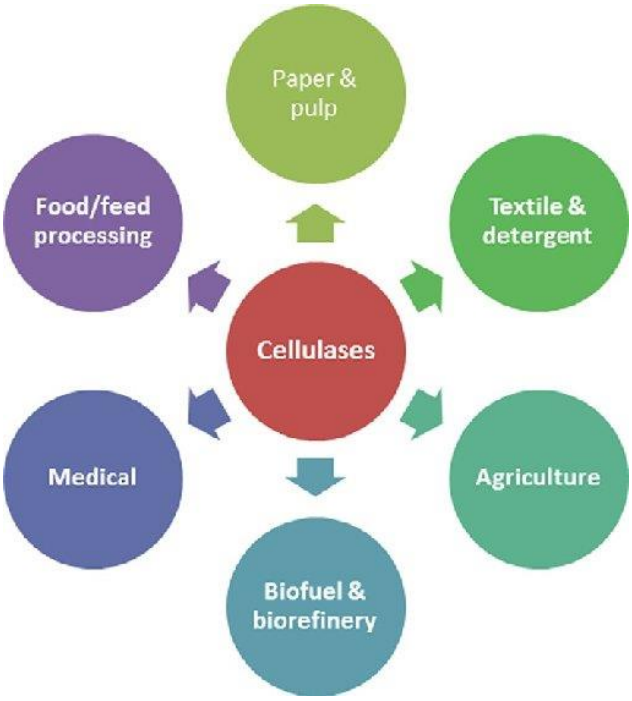


Fig 2.9 Applications of cellulases in different fields

2.7.1 Biorefinery

Cellulase breaks down LCB into simple sugars, which are fermented into bio-based fuel like bioethanol. The conversion of renewable LCB is mostly accomplished by cellulases. Bioprocessing of biomass by cellulolytic bacteria is projected to save 40% of the cost of the process. Currently, a number of countries have enacted policies relating to cellulosic ethanol, with the goal of shifting biomass resources away from starchy or cane sugars and toward cellulose-based materials [30].

2.7.2 Pulp and paper industry

Modification of fibre properties with a mixture of cellulases and hemicellulases is used in paper manufacturing factories to enhance drainage and vulnerability before or after pulp beating. Deinking various types of paper wastes is aided by cellulases alone or in combination with xylanases. Interestingly, numerous mills have investigated the use of cellulases to enhance drainage with the aim of growing production rate. Enzyme treatments remove part of the fines on the fibre surface, as well as dissolved and colloidal contaminants, which can cause serious drainage issues in paper mills [32,33].

2.7.3 Agriculture

Cellulases are commonly used in agriculture to improve crop growth and as a disease control agent. Combinations of cellulases and hemicellulases are commonly utilised for this purpose. Certain fungal cellulases have the ability to breakdown plant pathogen cell walls. There are numerous details concerning using bacteria to increase plant performance, such as plant growth-promoting rhizobacteria (PGPR). These bacteria are said to serve a key role in decreasing the use of artificial fertilisers, promoting plant development, and regulating possible plant infections and disease protection [25].

2.7.4 Food industry

Enhanced extraction, clarification and stabilisation procedures are needed for the production of fruits and vegetables juices. Cellulase is widely utilised as a macerating enzyme for the recovery of these juices. Cellulase is also utilised to enhance the cloud stability in pastes of tropical fruits like mango, peach and papaya. The texture, flavour, and aroma qualities of fruits and vegetables can be improved by infusing enzymes such as β-glucosidases into citrus fruits to reduce excessive bitterness [33]

CHAPTER 3: MATERIALS AND METHODS

3.1 Collection of soil sample

The soil sample was collected from Delhi Technological University campus. The sample collection site was selected in such a way that it had the possibility of containing cellulase producing bacteria. The site has a lot of dried plants and leaves on its surface. Soil was collected by digging 1-2 cm of the surface using a spatula.

Fig 3.1 Soil collection site

3.2 Serial dilution of the collected soil sample

Serial dilution is a method of reducing the concentration of microbes in a solution to a more useable level by performing a series of dilutions in a stepwise manner. To make the master plates of the soil samples, 1g of soil was dissolved in 10 mL of 0.9% saline solution. The sample was then serially diluted in sterile saline solution until a dilution of 10^{-7}. All the dilutions were then plated on LB agar (LBA) medium by spreading 100µL of the diluted samples. The petri dishes were incubated at 30ºC for 24-48 hours.

3.3 Streaking of specific colonies

Morphologically different colonies from 10^{-3} and 10^{-4} diluted plates were then picked up and streaked separately on LBA petri dishes containing 0.1% carboxymethyl cellulose (CMC). These were incubated at 30ºC overnight. The specific colonies picked and streaked using the 10^{-3} and 10^{-4} master plates were given different names- A_3, B_3, C_3, D_3, E_3, A_4, B_4 and C_4 respectively.

3.4 Screening of cellulase producing bacteria

Single colonies of A_3, B_3, C_3, D_3, E_3, A_4, B_4 and C_4 were picked and inoculated on LBA plates containing 0.1% CMC at 30ºC overnight. The diameter of each colony was measured after growth. Petri dishes were then stained with 1% Congo red for 20 minutes and washed off with 1M sodium chloride. The diameter of clear zone produced by colonies was measured and the difference between clear zone and isolated colony was calculated. The colonies producing the maximum zone of clearance were selected for future work.

3.5 Morphological analysis of potential cellulase producing isolates

The isolated strains were morphologically analyzed using Gram's staining and were observed under 100X magnification of light microscope. Staining was performed to find out whether the isolated strains were Gram-negative or Gram-positive.

3.6 Submerged culture of isolated colonies

Seed culture of A_3 isolated colony was prepared by inoculating a A_3 colony in 25mL LB broth containing 0.1% CMC. It was grown overnight in an incubator shaker at 30ºC and 120 rpm. 500µL of this seed culture was then used to inoculate 100mL of secondary culture. The secondary culture was incubated at 30ºC and 120 rpm. 2mL of this secondary culture was taken out and kept in microcentrifuge tubes after every 2 hours interval till 26 hours. These microcentrifuge tubes were then centrifuged at 10,000 rpm for 7 minutes. Pellet and supernatant were separated and kept in different tubes. Pellet and supernatant were stored at 4ºC for future use.

Fig 3.2 Submerged culture of A_3 isolate

3.7 Growth curve determination

The growth curve was determined by dissolving the pellet in 0.9% saline solution. Absorbance was measured at 600nm.

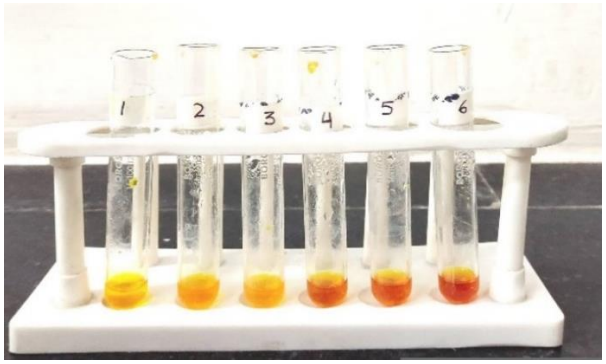
3.8 Total reducing sugar (TRS) assay

TRS assay was performed to determine the amount of reducing sugars present in the supernatant. Reducing sugar concentration was determined by plotting the standard glucose curve. For the plotting the glucose standard curve, a stock solution of 1mg/mL glucose was prepared. Different glucose dilutions were then prepared as shown in the following table-

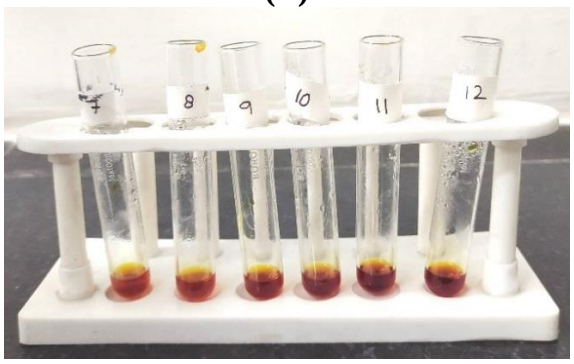
Table 3. Glucose dilutions prepared for plotting glucose standard curve

S.No.	Glucose stock concentration (1mg/mL)	Glucose working concentration (mg/mL)	Glucose working volume (µL)	Distilled water (µL)
1	1	0	0	1000
2	1	0.25	25	975
3	1	0.5	50	950
4	1	1	100	900
5	1	1.25	125	875
6	1	1.5	150	850
7	1	2	200	800

500µL of each dilution was taken in separate test tubes and mixed with 500µL of 3,5-dinitrosalicylic acid (DNS). The test tubes were boiled for 10 minutes. Different shades of brown color were developed in different test tubes which were determined spectrophotometrically at 540nm. The glucose concentrations and absorbance values were plotted on the X and Y axis respectively.



(A)



(B)

Figure 3.3 Glucose standard prepared by DNSA method; (A) 1-6 dilution tubes with glucose concentration of 0-1.5 mg/mL and (B) 7-12 dilution tubes with glucose concentration of 2-3.5 mg/mL

TRS assay was performed by mixing 500µL of supernatant collected at different time intervals with 500µL of DNS in different test tubes. These test tubes were then boiled for 10 minutes. Different shades of brown color were developed in different test tubes which were determined spectrophotometrically at 540nm. Absorbance values were used for the determination of unknown glucose concentration with the help of standard curve. A graph was then plotted between glucose concentration and time.

3.9 Endoglucanase assay using CMC

Endoglucanase assay is used to find out the activity of endoglucanase on cellulose. It is determined by plotting glucose standard curve as described in the previous step. After the preparation of glucose standard curve, 1% CMC was prepared in sodium citrate buffer of pH 5. This was treated as the substrate of the enzyme. Then 500µL of supernatant was mixed with 500µL of the substrate in a test tube, followed by incubation in water bath at 45ºC for 30 minutes. To stop the reaction, 3mL of DNS was added to the test tube. These test tubes were boiled for 10 minutes. These test

tubes were then boiled at 100ºC for 10 minutes. Different shades of brown color were developed in different test tubes which were determined spectrophotometrically at 540nm. Absorbance values were used for the determination of unknown glucose concentration with the help of standard curve. After the determination of glucose concentration, the following formula was used for the determination of enzyme activity. A graph is then plotted between enzyme activity and time.

$$\text{Enzyme activity} = \frac{\text{Product concentration} \times \text{Total reaction volume} \times \text{Dilution factor}}{\text{Molecular weight} \times \text{Enzyme volume} \times \text{Incubation time}}$$

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Soil collection site

The soil sample was collected from Delhi Technological University campus. The sample collection site was selected in such a way that it had the possibility of containing cellulase producing bacteria. The site has a lot of dried plants and leaves on its surface. Soil was collected by digging 1-2 cm of the surface using a spatula. The collected soil was stored in sealed plastic bags and kept at 4˚C until further use.

Fig 4.1 Collected soil sample

4.2 Serially diluted plates

The maximum colonies were obtained on 10^{-1} and gradually kept decreasing as the dilution was increased. The plates were observed and kept at 4˚C until further use.

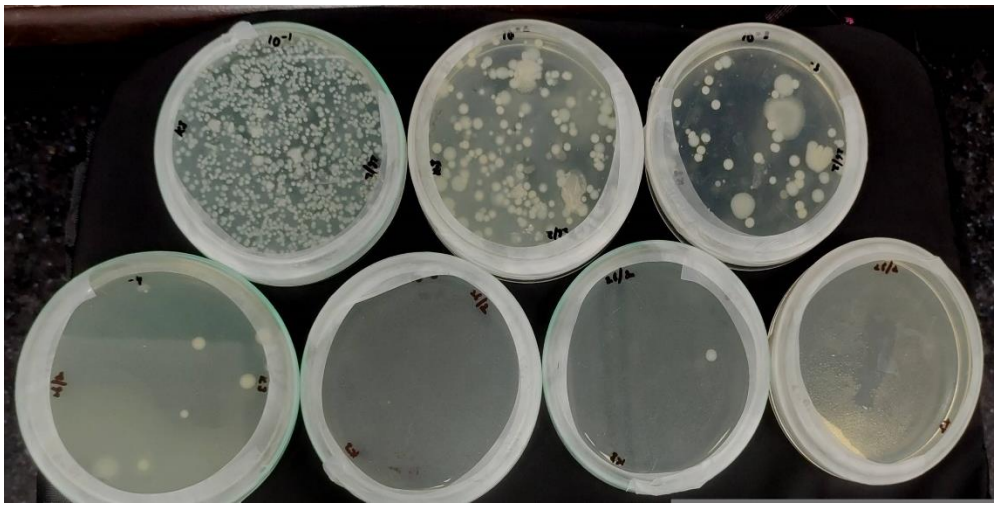


Fig 4.2 10^{-1} to 10^{-7} serially diluted plates after overnight incubation

4.3 Streaked plates of specific colonies

Morphologically different colonies were picked up from the serially diluted plates and streaked on the LB agar plates with 0.1% CMC. These were incubated at 30ºC for 24 hours.

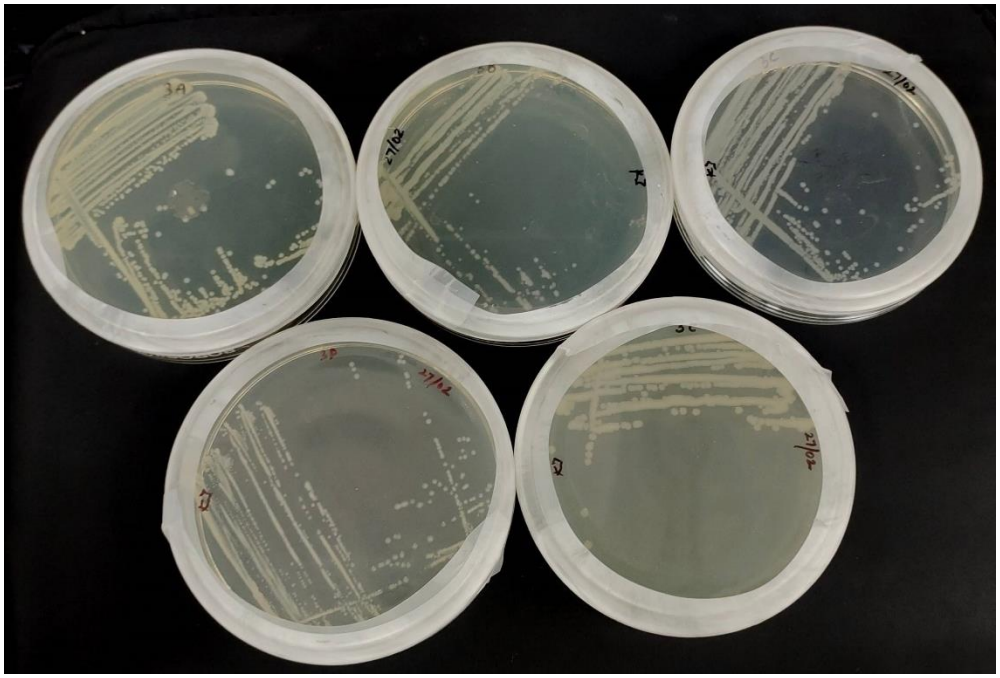


Fig 4.3 Streaked A_3, B_3, C_3, D_3 and E_3 plates after overnight incubation

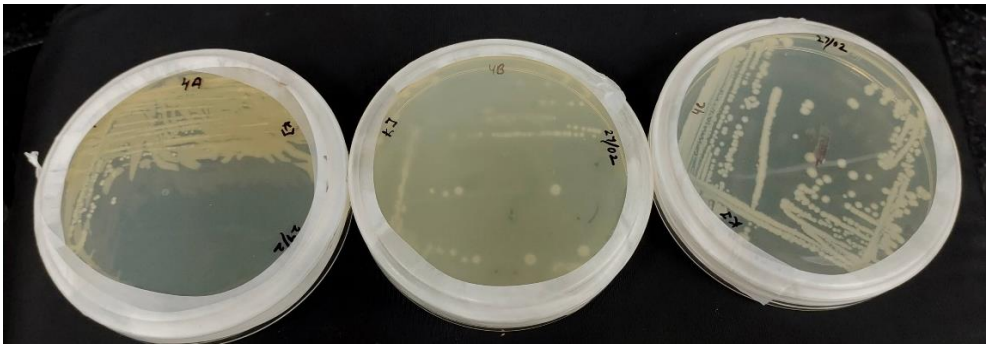


Fig 4.4 Streaked A_4, B_4 and C_4 plates after overnight incubation

4.4 Congo red staining of specific colonies

Single colonies were picked and inoculated on LB plates with 0.1% CMC. They were incubated at 30ºC for 24 hours. After growth, these plates were flooded with 1% Congo red and washed with 1M sodium chloride. The experiment was performed in replicates. A clear zone of hydrolysis was formed as shown in the following figures-

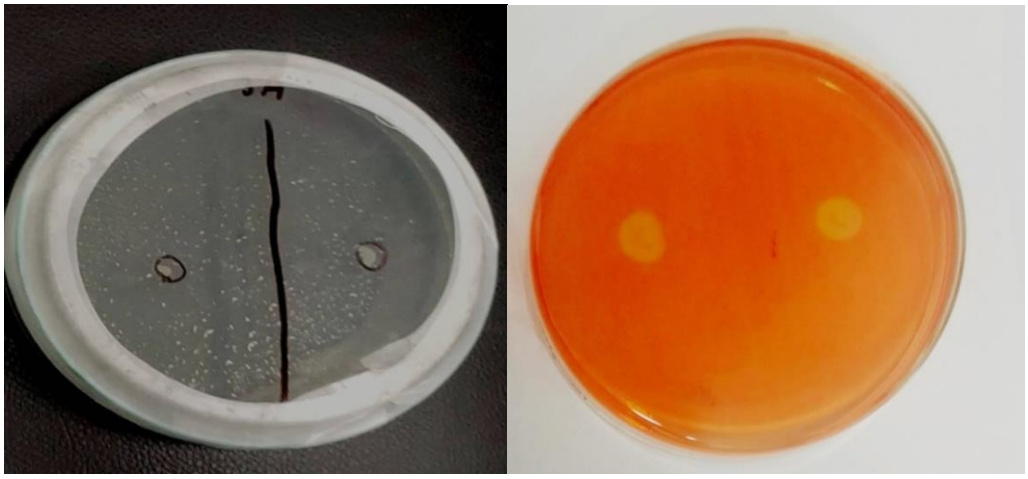


Fig 4.5 A_3 plates before and after congo-red staining

Clear zone was obtained in A_3 colonies

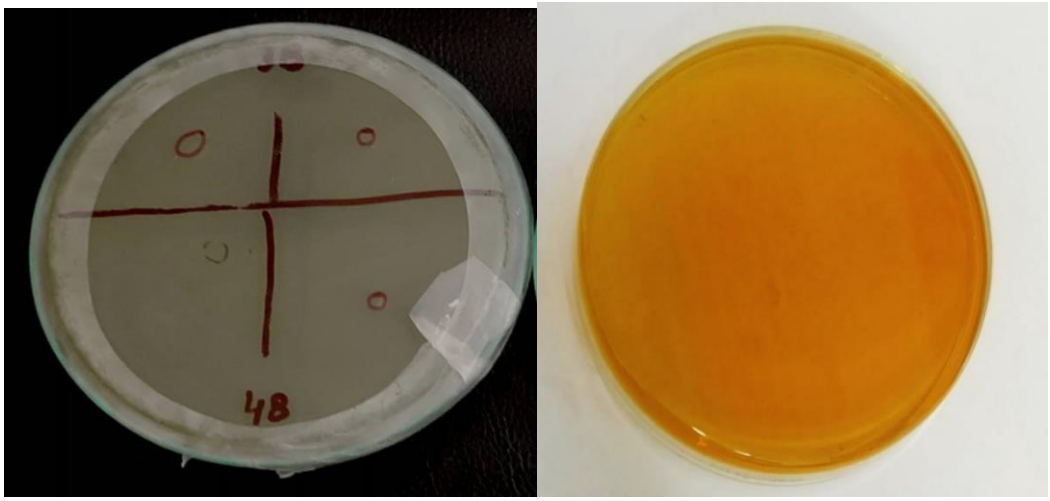


Fig 4.6 B_3 and B_4 plates before and after congo-red staining

No clear zone was obtained in B_3 and B_4 colonies.

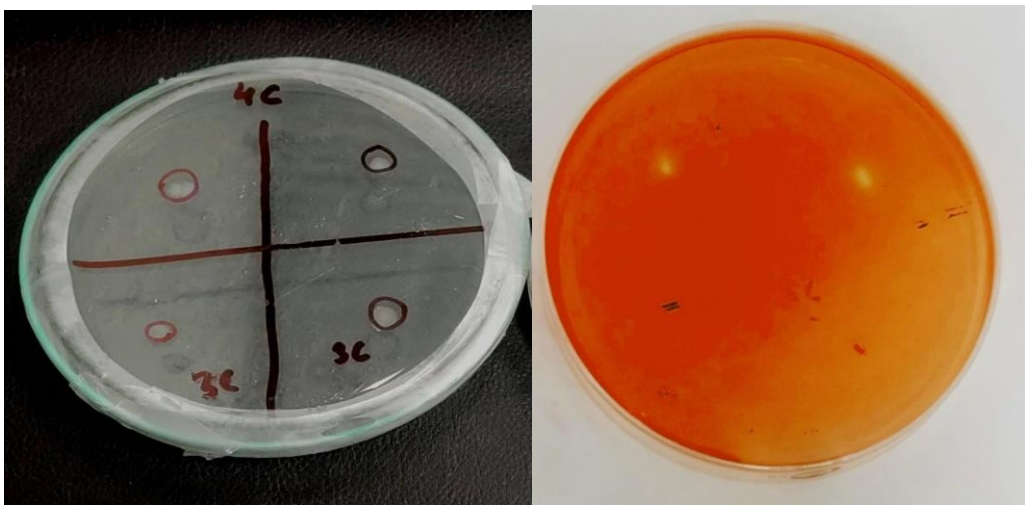


Fig 4.7 C_4 and C_3 plates before and after congo-red staining

Clear zone was obtained in C_4 colonies but not in C_3.

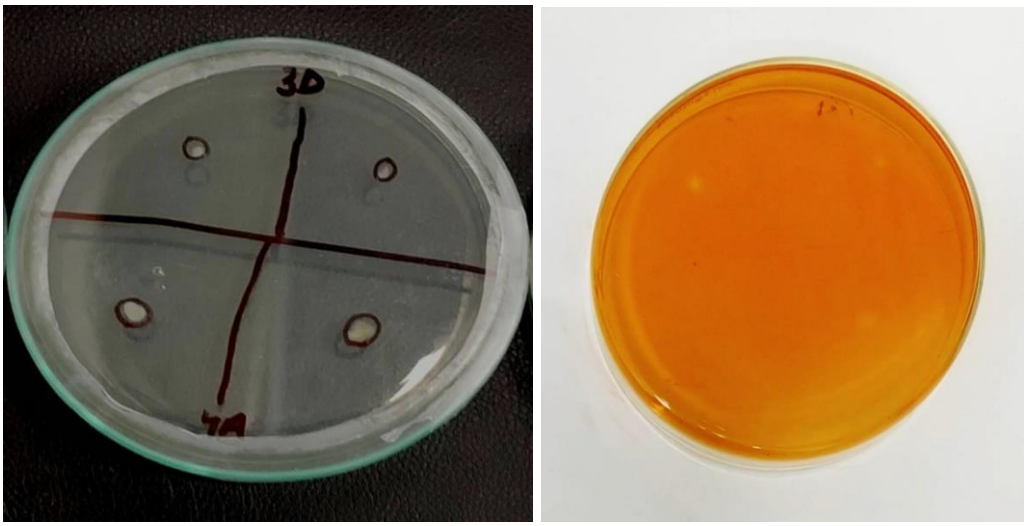


Fig 4.8 D_3 and A_4 plates before and after congo-red staining

Clear zone was obtained in both D_3 colonies and A_4 colonies.

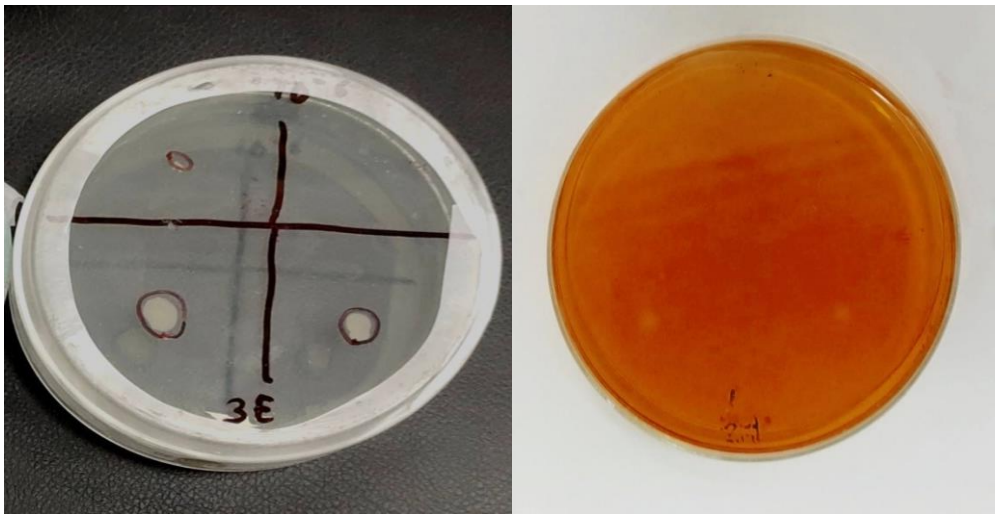


Fig 4.9 A_6 and E_3 plates before and after congo-red staining

Clear zone was obtained in E_3 colonies but not in A_6.

Colony diameter and clear zone diameter was calculated using a ruler. Colony diameter was then subtracted from the clear zone diameter to find out the colony showing maximum cellulase production.

Table 4. Colony diameter, clear zone diameter and their difference

Colony name	Colony diameter (cm)	Clear zone diameter (cm)	Colony diameter-clear zone diameter (cm)
A_3	0.5	1.4	0.9
B_3	0.6	-	-
C_3	0.5	-	-
D_3	0.7	0.8	0.1
E_3	0.8	1.1	0.3
A_4	0.4	0.5	0.1
B_4	0.3	-	-
C_4	0.9	1.3	0.4
A_6	0.4	-	-

The maximum clear zone forming colonies were selected for further analysis i.e., A_3, E_3 and C_4.

4.5 Gram's staining of potential isolates

A_3, E_3 and C_4 were analysed with the help of Gram's staining via 100X light microscope and the following results were obtained-

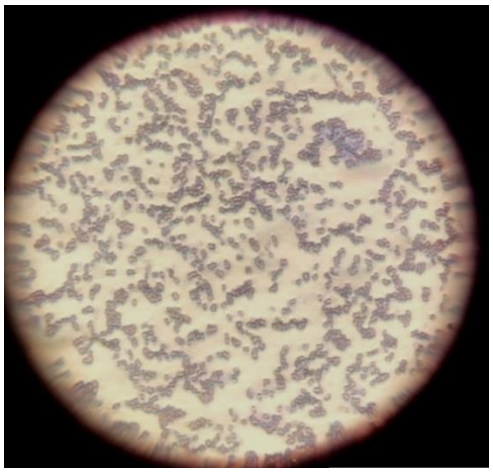


Fig 4.10 A_3 Gram's staining

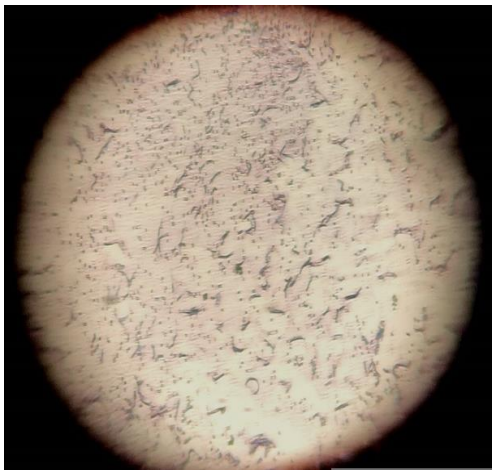


Fig 4.11 E_3 Gram's staining

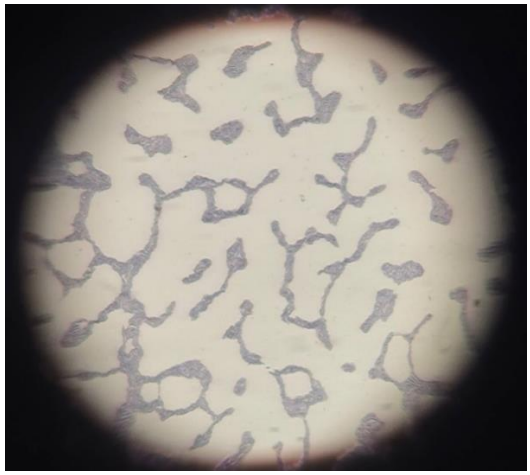


Fig 4.12 C_4 Gram's staining

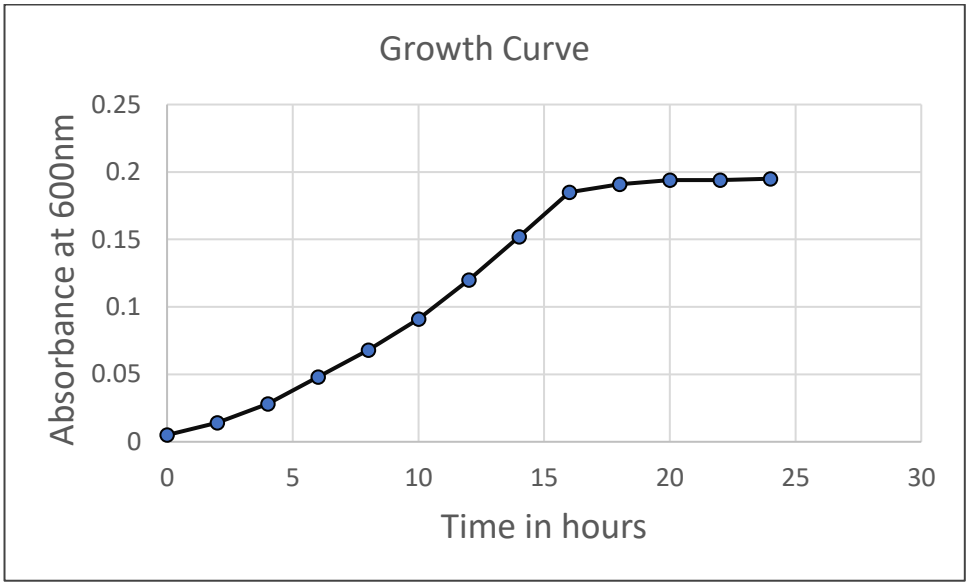
It was observed that A_3, E_3 and C_4 were Gram-positive bacteria. While A_3 was spherical (*Cocci*), E_3 was rod shaped (*Bacilli*) and C_4 was *Streptococcus*.

4.6 Growth curve of A_3 potential isolate

The following growth pattern was observed in A_3 by taking OD at 600nm after every 2 hours interval. The maximum growth was observed at 16 hours.

Table 5. Absorbance (600nm) taken at time interval of 2 hours

S.No.	Time (hour)	Absorbance (600 nm)
1	0	0.005
2	2	0.014
3	4	0.028
4	6	0.048
5	8	0.068
6	10	0.091
7	12	0.12
8	14	0.152
9	16	0.185
10	18	0.191
11	20	0.194
12	22	0.194
13	24	0.195



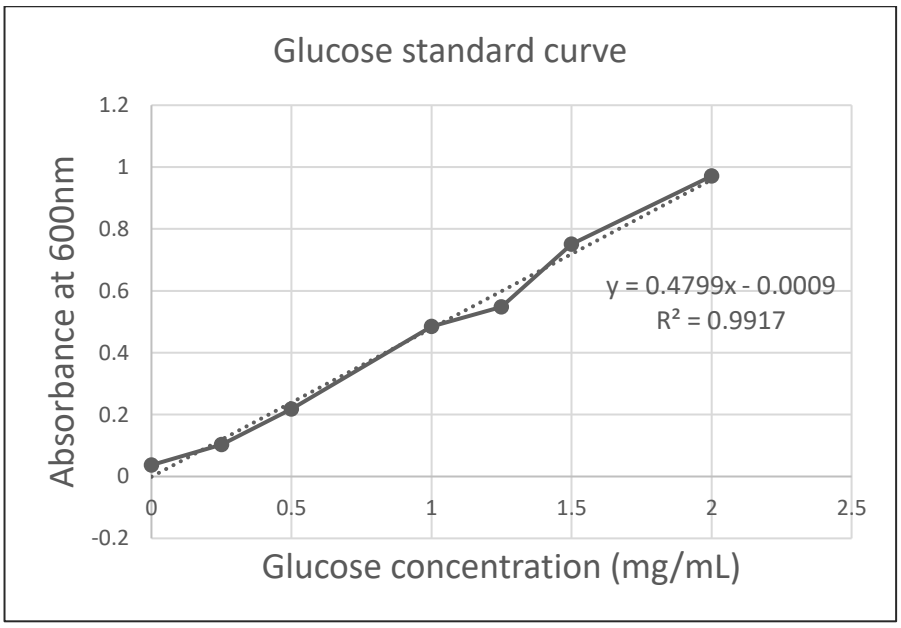
Graph 1: Growth curve of 3A bacteria prepared by plotting time vs absorbance

4.7 Glucose standard curve

The following absorbance was observed for the different glucose concentrations-

Table 6. Absorbance (540nm) with respect to glucose concentration

S.No.	Glucose concentration (mg/mL)	Absorbance at 540nm
1	0	0.037
2	0.25	0.103
3	0.5	0.218
4	1	0.485
5	1.25	0.548
6	1.5	0.751
7	2	0.971



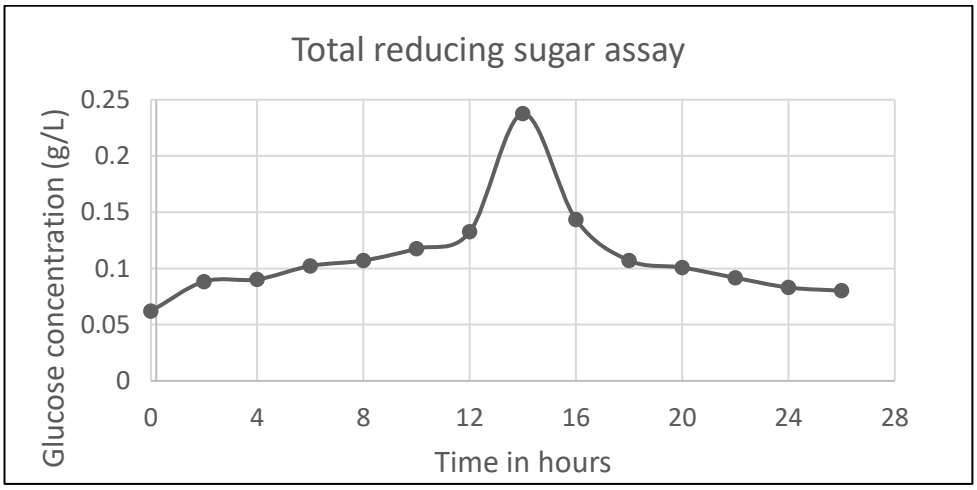
Graph 2: Standard glucose curve prepared by plotting glucose concentration vs absorbance

4.8 Total reducing sugar assay

TRS assay was used to find out the amount of reducing sugars present in the supernatants at different time intervals. Maximum concentration of reducing sugars was observed at 14 hours.

Table 7. Absorbance with respect to time and the corresponding glucose concentrations

S.No.	Time interval (Hours)	Absorbance (540nm)	Glucose concentration (g/L)
1	0	0.131	0.061945
2	2	0.186	0.08834
3	4	0.19	0.09026
4	6	0.215	0.102258
5	8	0.225	0.107057
6	10	0.247	0.117615
7	12	0.278	0.132493
8	14	0.497	0.237595
9	16	0.301	0.143531
10	18	0.225	0.107057
11	20	0.212	0.100818
12	22	0.193	0.0917
13	24	0.175	0.083061
14	26	0.169	0.080181



Graph 3: TRS assay graph prepared by plotting time vs glucose concentration

4.9 Endoglucanase activity assay

Endoglucanase activity assay was performed by using CMC as the substrate. The readings obtained were as follows. The glucose concentration found out using the standard curve was taken to calculate the enzyme activity using the following formula-

$$\text{Enzyme activity} = \frac{\text{Product concentration} \times \text{Total reaction volume} \times \text{Dilution factor}}{\text{Molecular weight} \times \text{Enzyme volume} \times \text{Incubation time}}$$

where,

Product concentration= amount of glucose liberated

Total reaction volume= 1mL (500µL substrate+500µL enzyme)

Dilution factor= 10

Molecular weight of glucose= 180g/mol

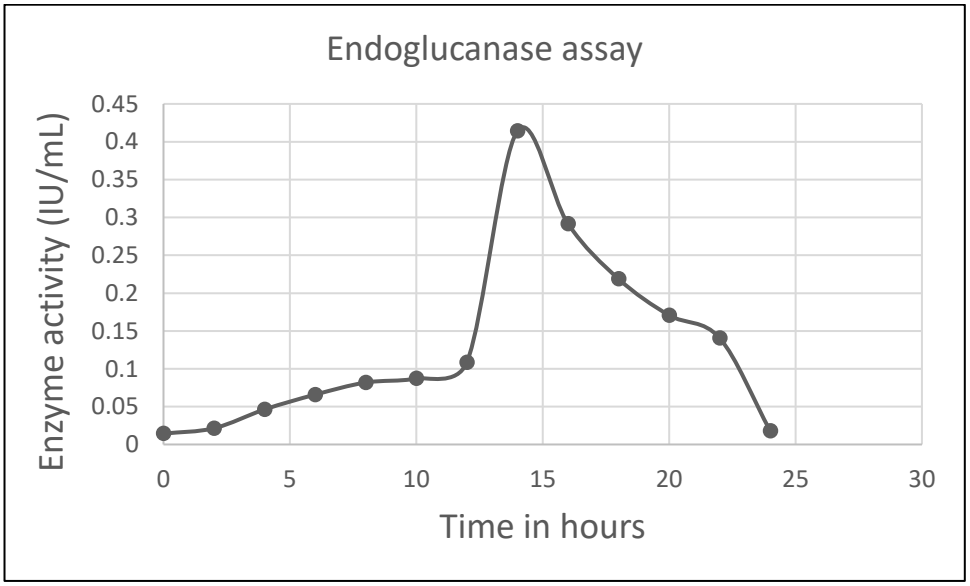
Enzyme volume= 500µL

Incubation time= 30 minutes

After calculating the enzymatic activities values, it was found that maximum activity of 0.4 IU/mL was observed at 14 hours.

Table 8: Absorbance with respect to time and their corresponding glucose concentration and enzyme activity

S.No.	Time interval (Hours)	Absorbance (540nm)	Glucose concentration (mg/mL)	Enzyme activity (IU/mL)
1	0	0.01	0.003874335	0.014349
2	2	0.014	0.005794012	0.021459
3	4	0.028	0.012512879	0.046344
4	6	0.039	0.017791988	0.065896
5	8	0.048	0.02211126	0.081894
6	10	0.051	0.023551017	0.087226
7	12	0.063	0.029310046	0.108556
8	14	0.235	0.111856127	0.414282
9	16	0.166	0.078741711	0.291636
10	18	0.125	0.059065029	0.218759
11	20	0.098	0.046107214	0.170767
12	22	0.081	0.03794859	0.14055
13	24	0.012	0.004834173	0.017904
14	26	0.01	0.003874335	0.014349



Graph 4: Endoglucanase assay graph prepared by plotting time vs enzyme activity

CHAPTER 5: CONCLUSION

LCB such as agricultural waste represents the majority of the total biomass present on the earth. LCB is a plentiful and renewable resource with significant potential in the production of second-generation biofuels and bio-based chemicals. LCB is comprised of cellulose, hemicellulose and lignin. LCB is recalcitrant in nature and therefore, its pretreatment is necessary for the structure modification and exposing of LCB fractions for better accessibility during enzymatic hydrolysis. After the LCB fractions are exposed, the fundamental step in the bioconversion of lignocellulosic biomass into bio-based products is based on the decomposition of cellulose by cellulase. Cellulases are enzymes that catalyse the hydrolysis of the linear glucose polymer cellulose's – β-1, 4 bonds. They belong to the glucoside hydrolases family of enzymes. Cellulases work by synergistic action of (i) endoglucanases, (ii) exoglucanases and (iii) β-glucosidases. Cellulases are produced by various microbes such as fungus, bacteria and actinomycetes. Even though fungus show the most efficient cellulase activities, there is growing interest in bacterial cellulase production due to the advantages it offers, such as faster growth rate, better specific activity and ease of genetic recombination. Thus, the discovery of a new and improved bacterial strain with increased cellulase productivity and activity could be a game-changer in the use of LCB for a variety of applications. In this study, cellulase producing bacteria was screened and isolated from soil samples. It was primarily screened by growing it on LBA and CMC media, followed by Congo-red staining. The potential isolate was then grown on liquid media containing LB broth and CMC. Its growth pattern was found out by plotting a growth curve and the enzyme activity was analysed by performing TRS assay and endoglucanase assay. It was observed that maximum cell biomass was achieved after 16 hours and maximum enzyme activity of 0.4 IU/mL was observed at 14 hours.

CHAPTER 6: FUTURE PROSPECTS

Enzymatic hydrolysis of LCB for the production of biofuels and other value-added products is a promising alternative to the conventional fuels. Lignocellulosic biomass can be exploited as a low-cost carbon source and thus, significantly reduce the cost of production. The key step in the bioconversion of lignocellulosic biomass into value-added products is based on the decomposition of cellulose by cellulase. In this study, we isolated cellulase producing bacteria and characterized it using TRS and CMC assay.

Further, strain characterization and identification can be carried out using 16s rRNA sequencing method. Filter paper assay and β-glucosidase assay can be performed to determine its exoglucanase and β-glucosidase activities. Further, its activity can be analysed on untreated, alkali-pretreated and acid-pretreated LCB. The process can then be optimized for parameters such as temperature, pH, incubation time etc. Various other techniques can also be applied which help in studying the structure, function and other characteristics of the cellulase. This will aid in the current work being carried out for the research and development towards enzymatic hydrolysis of LCB.

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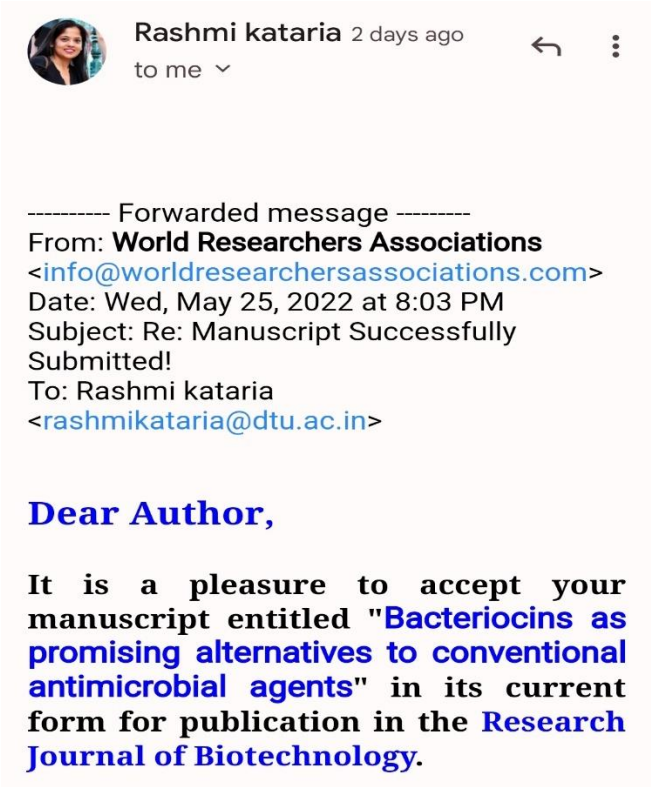
LIST OF PUBLICATIONS

My review paper titled, " **Bacteriocins as promising alternatives to conventional antimicrobial agents**", has been accepted in **Scopus indexed journal**: "Research Journal of Biotechnology" after the peer-review process. The proof of the same has been attached below.



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Bacteriocins as promising alternatives to conventional antimicrobial agents

Joshi Khyati, Kumar Pravir and Kataria Rashmi*

Department of Biotechnology
Delhi Technological University (DTU),
Bawana Road New Delhi 110042
*rashmikataria@dtu.ac.in; rashmikataria@gmail.com

Abstract

Bacteriocins are bacterially synthesised ribosomal antimicrobial peptides which hinder the growth of closely related or unrelated bacterial species. They are often compared to antibiotics due to their significant bactericidal properties. Reports suggest that almost all bacteria synthesise bacteriocins as a part of their innate immunity. Bacteriocin production is dependent on a number of process variables such as aeration, pH, temperature and the type of carbon and nitrogen source used. Bacteriocin applications are primarily focused on food preservation. However, resistance to conventional antimicrobial agents offers novel prospects for bacteriocin applications. Several recent studies have purified bacteriocins for food preservation, infection control, cancer therapy, peptic ulcers, etc. This paper summarises the classification of bacteriocins, their mechanism of action, factors affecting bacteriocin production, and the applications of bacteriocins as antimicrobial agents.

Keywords: Bacteriocins, Lantibiotics, Antimicrobial peptides, Bio-preservation, Gram-positive bacteria, Gram-negative bacteria

Introduction

Almost all life forms are known for their capability to synthesise antimicrobial peptides (AMPs)[27]. AMPs are low molecular weight compounds that play an important part in innate immunity of the host[46]. Bacterial AMPs can be synthesized either with or without the help of ribosomes[33]. AMPs that are synthesized with the help of ribosomes are known as bacteriocins[34]. Bacteriocins represent an important class of AMPs with several applications in the pharmaceutical and food sectors[9]. Bacteriocins are well characterized for their bactericidal activity and are often compared to antibiotics due to their ability to restrain the growth of closely related or non-related bacterial species[43,45].

Bacteriocins are synthesised by Gram-negative (-ve) and Gram-positive (+ve) bacteria. Lactic acid bacteria (LAB) represent the largest group of bacteriocin producing microbes[23]. LAB bacteriocins have received increasing interest amongst research groups due to their inherent tolerance against thermal and pH stress[35,44]. These features of bacteriocins make them suitable for various applications[18].

The potential health hazards of using chemical food preservatives have led to the quest for alternate methods of food preservation[22]. The utilization of bacteriocins as natural food preservatives has gained considerable attention in the past few years[39]. Besides food preservation, bacteriocins have received tremendous interest as promising antimicrobial agents[40]. Several LAB and other bacteriocins have been reported to be successfully utilised in food preservation and inhibition of pathogen growth[32].

The classification of bacteriocins, their mode of action, factors affecting bacteriocin production, and their uses in the food and pharmaceutical industries are covered in this overview.

Classification of bacteriocins

Bacteriocins are short, amphiphilic and hydrophobic peptides that show stability at elevated temperatures[3,25]. They can be classified using a variety of methods. The cell wall type of the producing organism is one such classification method. This classification method divides bacteriocins into two broad categories- Gram -ve and Gram +ve bacteriocins[40].

Bacteriocins from Gram -ve bacteria

There are four major classes of Gram -ve bacteriocins- colicins, colicin-like, microcins and phage-tail-like bacteriocins[47] (Fig. 1).

Colicins are bacteriocins synthesised by some strains of *Escherichia coli (E. coli)*[10]. Colicins have three distinct domains- an N-terminal translocation (T) domain, a core receptor-binding (R) domain and a C-terminal cytotoxic (C) domain. R domain facilitates the binding of bacteriocin to the outer cell membrane, T domain facilitates bacteriocin transport across the outer cell membrane with the help of translocator protein and C domain possesses the antibacterial activity. The bactericidal mechanisms of colicins are divided into 3 categories: 1) Pore-forming type colicins; 2) Nuclease type colicins; 3) Peptidoglycanase type colicins[45].

Colicin-like bacteriocins are typically synthesised by pathogenic bacterial strains such as *Pseudomonas aeruginosa, Klebsiella* spp. etc. They are comparable to bacteriocins synthesised by *E. coli* in terms of structure, size and function. Just like colicins, their antibacterial activity is attributed to formation of pores or action of nucleases[17,40].

Microcins are low-molecular weight, highly stable peptides produced by *Enterobacteriaceae family*. Microcins have 2 sub-classes, based on post-translational modification and the sequence of leader peptides. Class I microcins include low molecular weight peptides (< 5kDa) with complex post-translational modifications. They inhibit vital bacterial enzymes like RNA polymerases and DNA gyrases. Microcins B17, C7-C51 and J25 are some examples of class I microcins. Class II microcins include longer plasmid

peptides (5-10kDa) with no post-translational modifications. For example, MccV, MccL and Mcc24. Class II microcins also include linear peptides encoded in chromosome that contain an iron-carrier at the C-terminal. For example, microcins mH47 and G47. They work as antimicrobial agents by pore formation in the target cell membrane[47].

Phage tail-like bacteriocins are distinct set of bacteriocins synthesised by *Enterobacteriaceae*, *Vibrionaceae*, and *Pseudomonadaceae* families. Phage tail-like bacteriocins are high molecular weight particles that are considered to be defective bacteriophages. It is predicted that they have originated by several successive mutation of temperate phages[42].

Bacteriocins from Gram +ve bacteria

There are 3 major classes of Gram +ve bacteriocins- Class I, II and III[45] (Fig. 2).

Class I bacteriocins, also called lantibiotics, are short peptides (<5 kDa) with atypical amino acids such as lanthionine. Atypical amino acids are produced as a consequence of post-translational modifications. Class I bacteriocins has 3 subclasses: class Ia, Ib and Ic. Class Ia contains positively charged, hydrophobic and elongated peptides, for example, nisin. Class Ib consists of negatively charged or neutral globular peptides, for example, subtilin. Class Ic consists of sulfur-to-α-carbon-containing peptides[19,26].

Class II bacteriocins are medium-sized peptides (<10 kDa) with the unique property of heat tolerance. It is positively charged and has unmodified non-lanthionine. Class II bacteriocins have five subclasses: class IIa, IIb, IIc, IId and IIe. Bacteriocins of class IIa have a canonical arrangement of YGNGVXaaC at the N-terminal and are active against *Listeria* spp.; for example, pediocin PA-1. Bacteriocins of Class IIb make use of 2 different unaltered peptides to form a complete functional portion composite; for example, lactacin F and ABP-118. Bacteriocins of Class IIc are circular peptides; for example, enterocin AS-48 and carnocyclin A. Bacteriocins of Class IId are single and linear peptides; for example, lactococcin A. Bacteriocins of Class IIe are serine-rich at the carboxy terminal with siderophore-type post-translation modification; for example, microcin E492[45].

Class III bacteriocins are heat-sensitive, long peptides (>30 kDa). They have 2 subclasses: class IIIa and IIIb. Bacteriocins of Class IIIa consist of proteolytic enzymes that degrade the peptidoglycan present in Gram-positive bacteria; for example, lysostaphin. Class IIIb bacteriocins consist of non-lytic proteins such as Helveticin J. Helveticin J is an antimicrobial protein produced by *Lactobacillus helveticas* that acts against closely related species of Lactobacillus[21].

The de-established Class IV bacteriocins consist of bulky complexes of protein and distinct macromolecules. Class IV member peptides have been re-classified as bacteriolysins, leaving only 3 classes of gram positive bacteriocins[32] (Table 1).

Mechanism of action

Bacteriocins are well recognised for their capability to restrain microbial growth. Bacteriocins exhibit their inhibitory action using various mechanisms such as 1) alteration of enzyme activity; 2) restricting the germination of spores; 3) inactivation of the anionic carrier; 4) formation of pores in the cell membrane[38].

It is reported that nisin, which is a class I bacteriocin, has a dual working mechanism. It has the ability of binding with lipid-II, the key carrier for peptidoglycan transport. Binding of nisin with lipid-II blocks proper cell wall biosynthesis, causing cell demise. Moreover, it can interact with lipid-II to initiate the action of membrane insertion and form apertures in the cell membrane[34]. Lactococcin A, which is a class IId bacteriocin, makes use of receptors called mannose phosphotransferase system (Man-PTS), causing unrestricted opening of channels, leading to pore formation in the bacterial membrane[40] (Fig. 3).

Gram -ve bacteriocins inhibit bacterial growth by targeting the DNA, RNA and protein metabolism[34]. For example, MccB17 crosses the outer and inner membrane using OmpF porin and SbmA peptide transporter respectively. After crossing both the membranes, it inhibits the action of DNA gyrase. Another bacteriocin, MccJ25 crosses the outer and inner membrane via FhuA receptor and TonB+SbmA respectively. Once it enters the cell, it inhibits the action of RNA polymerase. MccC7-C51 crosses *E. coli* outer and inner membrane using OmpF porin and YejABEF transporter. Once it enters the cell, it blocks mRNA synthesis[13] (Fig. 4).

Factors affecting bacteriocin production

Bacteriocin production is influenced by various process parameters like aeration, temperature, pH and the type of carbon and nitrogen source used[1].

Lactobacillus sakei subsp. *sakei* 2a is a bacteriocin producing LAB. It is capable of inhibiting *Listeria monocytogenes* growth[14]. Malheiros et al[30] studied the effect of glucose and tween 20 supplementation in Man, Rogosa, and Sharpe (MRS) broth on bacteriocin synthesis in *Lactobacillus sakei*. It was found that addition of glucose and tween 20 to culture media and incubation at 25.0°C or 30.0°C and initial pH of 5.0 or 5.5 resulted in highest bacteriocin yields. Azevedo et al[14] reported that the combination of sucrose, insulin and glucose in MRS media stimulated bacteriocin production in *Pediococcus pentosaceus* ATCC 43200. However, the complete replacement of glucose by sucrose or insulin almost entirely supressed the bacterial growth, suggesting the glucose is the favoured carbon source of this strain. Malathi and Selvakumar[29] reported that maximum nisin production by *Lactobacillus lactis* MTCC 440 was observed at 30ºC, pH 5, 1% salinity when glucose and peptone were used in the media at an incubation period of 24h. Elayaraja et al[16] found that bacteriocin production in *Lactobacillus murinus* AU06 increased up to 4.74 times when it was incubated at 35.0°C and pH 6. *Lactobacillus viridescence* bacteriocins are highly effective against food borne microbes and thus, is a potent candidate for the preservation of various food items[24]. Sure et al[24] reported that highest bacteriocin production in *Lactobacillus viridescence* was observed when it was grown at 37.0°C for 48 hours in MRS broth of pH 7. Abo-Amer[2] investigated the effect of various process parameters on bacteriocin production in *Lactobacillus acidophilus*. Maximum bacterium growth was achieved at 37°C whereas maximum bacteriocin production was attained at 30°C. The use of synthetic media and other nutritional components increases the overall cost of bacteriocin production. Utilization of agricultural waste as substitutes of carbon and nitrogen sources can enhance the economic viability of the process[36]. Agro-industrial by-products were used by Bali et al[6] to produce bacteriocin in *Enterococcus faecium*. It was observed that maximum bacteriocin activity was achieved when medium containing whey was used at 40ºC and 100 rpm[5].

Applications

Various recent studies have recognized bacteriocins for applications in food preservation, treatment of infectious diseases, cancer therapy and maintaining human health[45].

Food preservation

Preservatives are added to extend the food shelf life by delaying microbial growth. Presently, most of the commercial preservatives are chemical in nature. The long-term use of these chemical preservatives has serious ill effects on the human body[45]. Keeping the above problem in mind, bio-preservatives are in high commercial demand[37]. Since bacteriocins have no taste, odour and colour, they can be utilised as bio-preservatives. Moreover, most of the bacteriocins show stability at high temperature, low pH and across a widespread salt concentration range[41]. Bacteriocins involved in food applications can be classified into 3 categories: 1) Partially purified bacteriocins; 2) Bacteriocins as crude fermentate; 3) Bacteriocin-producing protectice cultures[12]. Presently, nisin is used as a preservative and is commercialized as NisaplinR, ChrisinR and DelvoR Nis. It is used to control clostridia and is widely utilized in the dairy industry[41]. Another

bacteriocin, pediocin PA1/AcH has been demonstrated to efficiently reduce listeria populations in whole milk, ice cream mix, sausage mix and fresh and ground beef[8].

Treatment of Peptic Ulcer

It has been observed that anaerobic *Helicobacter pylori* is found in excessive amounts in peptic ulcer patients. Bacteriocins can be used for the formulation of therapies intended to treat such ulcers. Since bacteriocins are deactivated by digestive proteases, they do not alter the gut normal flora. Several class I bacteriocins have been reported to showcase bactericidal activity against *Helicobacter pylori.* For example, nisin A, JW3, NK24, lacticins A164, leucocin K and pediocin PO2. Some class II bacteriocins have also been tested in-vitro. For example, bulgaricin BB-18 and lactacin F. The bacteriocins can decrease the side effects of chemical therapy when used along with antibiotics[31,34].

Spermicide action and women care

Among various population control methods, contraceptive chemical spermicides are also an option. Chemical spermicides have various side effects such as vaginal and urinary tract infection. Bacteriocins have the ability to affect sperm motility, thus, are potential natural spermicides. For example, fermenticin HV6b and lactacin have been reported to cause reduction in the human spermatozoa motility. They work by coiling the sperm tails and damaging the cells in a way that their moment is restricted. Fermenticin HV6b has also been reported to kill vaginal pathogens like *Gardnerella vaginalis.* Other bacteriocins such as lactocin 160 produced by *Lactobacillus rhamnosu* and SB83 produced by *Pediococcus pentosaceus* SB83 have displayed effective action against *Gardnerella vaginalis, Mobiluncus, Peptostreptococcus* and *Prevotella bivia* spp.[28].

Infection control

There is a vital need for the development of novel drug molecules due to increasing number of antibiotic-resistant microorganisms. Bacteriocins are effective against numerous pathogens and thus, are suitable alternatives to antibiotics. For example, nisin has been reported to show its action against antibiotic vulnerable as well as resistant strains of *Staphylococcus aureus*. Other bacteriocins evaluated to show their effect against methicillin resistant *Staphylococcus aureus* (MRSA) strains are Laterosporulin10, microbisporicin, NVB333 and mersacidin. Gallidermin and epidermin synthesised by *Staphylococcus gallinarum* and *Staphylococcus epidermidis* respectively, are reported to be effective in skin infection treatment. Some bacteriocins have been also been stated to be effective against tuberculosis causing *Mycobacterium tuberculosis.* For example, griselimycin is effective in treating tuberculosis infected mice *in vivo*[6,20,21,41].

Cancer therapy

Bacteriocins display cytotoxic effect against cancer cells and thus, can be exploited as tools for the production of novel anticancer drugs[11]. Their anticancer mechanism is based on apoptosis, depolarization of cell membrane, inhibition of cell growth and blockage of angiogenesis[4]. Pore-forming bacteriocins such as colicin A and E1, for example, inhibit 1 human fibroblast cell line, MRC5, as well as 11 tumour cell lines. In another trial, nisin was used to treat three cases of head and neck squamous cell carcinoma[45].

Discussion

Bacteriocins are bacterially produced AMPs synthesized with the help of ribosomes[34]. They are low molecular weight compounds that perform a role in the innate immunity of the cell[46]. They constrain the

growth of closely related or unrelated bacterial species and therefore, are often compared to antibiotics[43,45]. Bacteriocins are produced by both Gram -ve bacteria such as *Klebsiella* spp., *E. coli*, *P. aeruginosa* and Gram +ve bacteria such as *Lactobacillus helveticas, Bacillus spp*., *Enterococcus faecalis*. The most studied group for bacteriocin production is LAB[23]. Bacteriocins show high bactericidal activity against Gram-positive bacteria but their bactericidal activity is not very efficient against Gram -ve bacteria because of the presence of outer membrane[38]. There are several classes and sub-classes of bacteriocins, among which nisin is the most intensively studied[45,47]. Bacteriocins are primarily used for food preservation[41]. Nevertheless, the emerging concern over the rise of antibiotic resistance bacteria offers novel prospects for bacteriocins' applications in the field of pharmaceutical sector[40]. Presently, bacteriocins are utilized for various purposes such as food preservation, treatment of peptic ulcers, spermicide action, infection control and cancer therapy[6,8,11,20,21,28,31,34,41,45]. Over the last decade, there has been a rise in the number of bacteriocin in-vivo assessments, but clinical applications need further emphasis for their potential use as antimicrobial agents.

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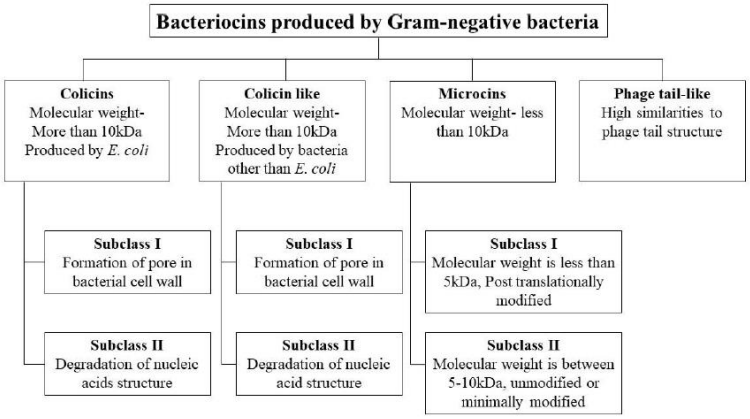

Fig. 1: Classes and subclasses of bacteriocins produced by Gram -ve bacteria

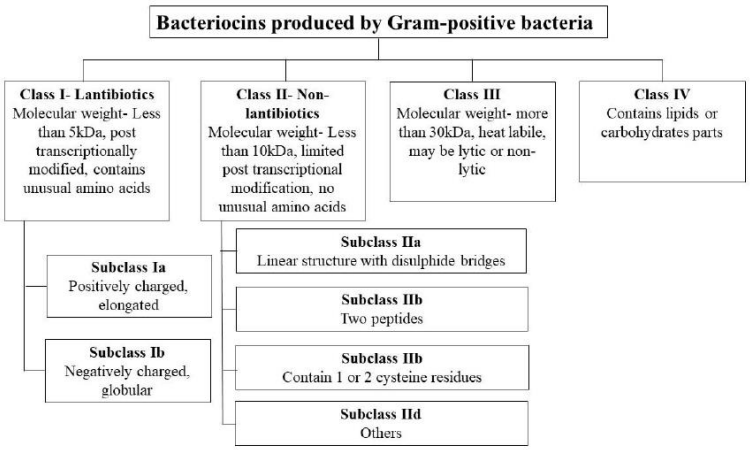

Fig. 2: Classes and subclasses of bacteriocins produced by Gram +ve bacteria

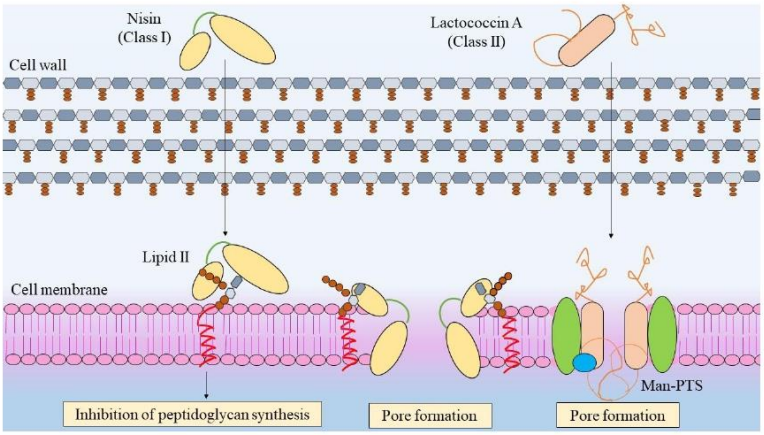

Fig. 3: Action of bacteriocins on Gram +ve bacteria

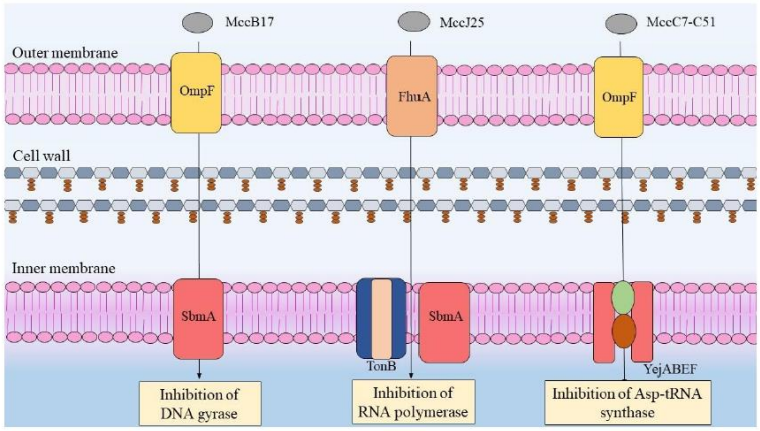

Fig. 4: Action of bacteriocins on Gram -ve bacteria

Table 1 Bacteriocins, their class and targets molecules

Bacteriocin	Class	Target molecule
Nisin, bovicin HJ50, haloduracin	Lantibiotics	Lipid II
Cinnamycin	Lantibiotics	Phosphatidylethanolamine
Bottromycin A2	Bottromycins	50S ribosomal subunit
Streptomonomicin	Lasso peptides	WalR response regulator
Sublancin 168, glycocin F	Glycocins	Glucose-phosphotransferase system
Garvicin ML	Head-to-tail cyclized bacteriocins	Maltose ABC transporter
Lactococcin A, garvieacin Q	Other linear bacteriocins	Mannose-phosphotransferase system
Plantaricin JK	2-peptide bacteriocins	APC transporter
Lactococcin 972	Other linear bacteriocins	Lipid II
Thiostrepton, nosiheptide, micrococcin	Thiopeptides	50S ribosomal subunit
GE2270A	Thiopeptides	Elongation factor TU
Lassomycin	Lasso peptides	ClpC1 ATPase
Sublancin 168	Glycocins	MscL mechanosensitive channel
Pediocin PA-1, enterocin 1071, leucocin A	Bacteriocins with YGNG-motif	Mannose-phosphotransferase system
Lactococcin G, enterocin 1071	Two-peptide bacteriocins	Undecaprenyl pyrophosphate phosphatase
LsbB, enterocin K1	Leaderless bacteriocins	Zn-dependent membrane metallopeptidase
Diffocin, monocin	Tailocins	Lipopolysaccharides

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