SCREENING, AND OPTIMIZATION OF CELLULASE-PRODUCING BACTERIA ISOLATED FROM DIFFERENT ENVIRONMENTAL SAMPLES USING LIGNOCELLULOSIC BIOMASS AND THEIR UTILIZATION IN BIOETHANOL PRODUCTION

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RECOMMENDATION

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With deep appreciation and sincere thanks,

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Glossary Acronyms

(Lists of abbreviation words)

µmol	**Micromole**
ng	**Nanogram**
µL	**MicroLiter**
℃	**Degree Centigrade**
APS	**Ammonium per Sulfate**
BLAST	**Basic Local Alignment Search Tool**
Bp	**Base pair**
BSA	**Bovine Serum Albumin**
CBB-G250	**Coomassie Brilliant Blue-G250**
CDBT	**Central Department of Biotechnology**
CMC	**Carboxy Methyl Cellulose**
CMCase	**Carboxymethyl Cellulase**
CTAB	**Cetyl Trimethyl Ammonium Bromide**
DNA	**Deoxyribonucleic Acid**
DNS	**Dinitrosalicylic acid**
DO	**Dissolved Oxygen**
Endo-G	**Endoglucanase**
Exo-G	**Exoglucanase**
G	**Gram**
IU	**International Unit**
K_m	**Michaelis Constant**
L	**Litre/litres**
M	**Metre**
Mg	**Milligram**
ML	**Milliliter**
MRVP	**Methyl Red Voges-Proskaur**
MW	**Molecular Weight**

NA	**Nutrient agar**
O/F	**Oxidative/Fermentative**
PCR	**Polymerase Chain Reaction**
PDA	**Potato dextrose agar**
rDNA	**Ribosomal DNA**
SDS-PAGE	**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis**
SIM	**Sulphur Indole Motility**
TE	**Tris EDTA**
TEMED	**Tetra methyl Ethylenediamine**
TSIA	**Triple Sugar Iron Agar**
v/v	**Volume/Volume**
w/v	**Weight/Volume**
V_{max}	**Maximum velocity**

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ABSTRACTS

This thesis investigates eco-friendly alternatives to fossil fuels in response to the demand for sustainable energy. It focuses on bioethanol production from lignocellulosic biomass, emphasizing the isolation, screening, and optimization of cellulase-producing bacteria. Total 16 cellulose-degrading bacteria were isolated from garden soil, plant residues, rotten wood, and Cow dung of Kirtipur, Chovar and Lainchour sites of Kathmandu, Nepal. Cellulolytic bacteria were screened primarily by plate hydrolysis and further confirmed by well diffusion method along with Congo red staining followed by NaCl treatment. Out of 12 pure bacterial isolates, 10 showed good cellulolytic activity. Notably, the cow dung-derived U1C4 isolate displayed the highest cellulolytic potential and was selected for further studies. This isolate, identified as *Bacillus cereus spp.* strain U1C4 was confirmed through morphological, biochemical, and phylogenetic analyses. Further investigations involved utilizing various lignocellulosic substrates as carbon sources including, *Saccharum spontaneum*, rice straw, wheat straw, barley straw, and wood for cellulase production after hot water pretreatment of samples. *Saccharum spontaneum* showed the highest cellulase production with cellulolytic activity 0.488 ± 0.021 IU/mL by U1C4 strain. The crude enzyme was purified using $(NH4)_2SO_4$ precipitation, dialysis, and chromatography, resulting in a 5.4% recovery rate, 6.34-fold purification, and 4.693 U/mg specific activity. The purified enzyme displayed a molecular weight of 50 kDa on SDS-PAGE. Upon kinetic analysis, the purified CMCase enzyme showed Km and Vmax values of 5.16 mg/mL and 33.22 µmol/ml/min, respectively. The optimum temperature and pH for maximum cellulase production were obtained as 35°C and 8 respectively including *Sacchharum spontaneum* (2%) and peptone (1%) as carbon and nitrogen sources for enhancing maximum cellulase production capabilities. Upon scaling up to a 3.5L production medium, the optimized *Bacillus sp*. (U1C4) showed improved enzyme production in an Electrolab fermenter 360 compared to a 250 mL conical flask. The CMCase activity reached the maximum of 0.595 ± 0.021 U/mL in the fermenter on 4th day, surpassing the activity of 0.484 ± 0.04 U/mL observed in the conical flask. Ethanolic fermentation was done using a well-optimized CDBT-2 strain of *S. cerevisiae* after the saccharification of *S. spontaneum* using U1C4 strain for 7 days and the highest ethanol concentration, 10.73± 0.045 mg/mL was achieved on 3rd day of the fermentation process. Additionally, the efficiency of ethanol production, as measured by the yield in relation to the production of reducing sugars, reached an impressive 82.65%. This yield was obtained by utilizing 17.709 mg/mL of reduced glucose during fermentation. These findings demonstrate that ethanol can be produced from agricultural waste, highlighting the potential of this process to generate wealth from waste.

Keywords: Bioethanol, *Bacillus sp.,* Cellulase, Lignocellulose, *Saccharum spontaneum*, Fermentation, CMCase, Pretreatment.

CHAPTER 1

INTRODUCTION

1.1 Background

In recent years, there has been a growing focus on bioenergy generation as a sustainable solution to address future energy challenges. This interest stems from various factors such as the anticipated energy crisis, increasing energy demands, soaring fuel prices, and mounting concerns about global warming caused by greenhouse gas emissions. Biofuels have already become a significant energy source for more than half of the world's population, constituting over 90% of energy consumption in developing countries (Msangi et al., 2007). What makes biofuels attractive is their relatively neutral carbon balance. Unlike petroleum-derived fuels like gasoline, diesel, or kerosene, biofuels derived from lignocellulosic materials have a carbon footprint that is close to neutral. This means that the amount of carbon dioxide released when these biofuels are burned is roughly equivalent to the carbon dioxide absorbed during the growth of the plant material used to produce them. Consequently, biofuels are gaining popularity in the current context of global warming concerns (Graves et al., 2011).

Among the various biofuels, bioethanol holds great potential due to its clean-burning properties and compatibility with existing fuel infrastructure. Bioethanol can be derived from a variety of renewable sources abundant in carbohydrates. These sources can be broken down into fermentable sugars, which are then converted into ethanol. There are three primary types of feedstock used for bioethanol production: First-generation bioethanol-produced from sucrose and starch-rich crops like cereals, sugarcane, corn, and similar plants, Second-generation bioethanol-utilizes lignocellulosic biomass as its source and Third generation bioethanol-Microalgae serve as the feedstock for this type of bioethanol (Bušić et al., 2018).

First-generation bioethanol dominates the global production of over 27,000 million gallons (over 102,060 million liters) of bioethanol, as of 2021. The United States of America and Brazil are the clear frontrunners, responsible for nearly 85% of the world's bioethanol output. The United States primarily produces bioethanol from corn, while Brazil relies on sugarcane as its main feedstock (Figure 1). However, the increasing production of first-generation bioethanol, coupled with a growing population, raises concerns about its long-term sustainability. These concerns include potential threats to global food and feed security, the need for land and water resources, and the possibility of soil contamination from distillation residues. As a result, extensive research is being conducted on alternative technologies for second-and third-generation bioethanol production (Broda et al., 2022).

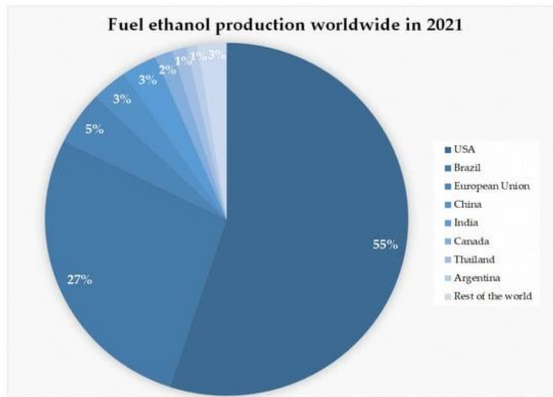


Figure 1: World's top leaders in bioethanol production in 2021 (Source: (Broda et al., 2022))

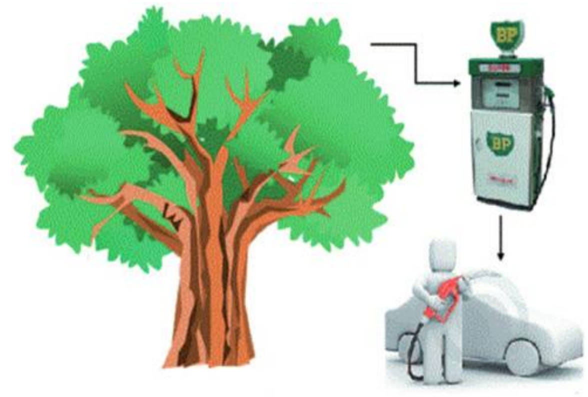


Figure 2: Second generation fuel (Source: (Hakeem et al., 2015))

Second-generation biofuels, also known as advanced biofuels, are produced from sources such as lignocellulosic biomass, nonfood crops and agricultural and forest residues, and industrial wastes. The primary goal is to maximize the potential of these agricultural byproducts and underutilized crops(biomass) to produce biofuels in an environmental friendly and economically liable manner(Suali & Suali, 2023). However, the efficient conversion of these lignocellulosic biomass, the most abundant and widely available renewable resource, into bioethanol is a complex process. Lignocellulosic biomass is composed of cellulose (33% to 51%), hemicellulose (19% to 34%), and lignin (20% to 30%), and its degradation requires multi-step enzymatic hydrolysis to break down the polysaccharides in cellulose and hemicellulose into monomeric sugars. Cellulases, a class of enzymes that specifically hydrolyze cellulose into fermentable sugars, play a crucial role in this process (Bušić et al., 2018).

Cellulases are a group of three enzymes: endo-1, 4-β-glucanase (Endoglucanase), exo-1, 4- β-glucanase (Exoglucanases), and β-glucosidase, which work together to break down cellulose into soluble sugars and glucose. Endoglucanases randomly attack the cellulose crystalline structure, breaking the glucose chains into shorter ones. Exoglucanases then act on the exposed ends of these chains, releasing cellobiose and some glucose. Finally, β-glucosidases complete the process by breaking cellobiose and cello-oligosaccharides into glucose molecules. Cellulases are produced by microorganisms including fungi, bacteria, and actinomycetes. Fungi are commonly used for commercial cellulase production due to their high enzyme activity, but bacteria, such as *Bacillus, Clostridium, Cellulomonas, Ruminococcus*, and *Alteromonas* show potential due to their faster growth rate, heat stability, and genetic manipulability (Gaur & Tiwari, 2015). Cellulases find broad applications in industries, particularly in biofuel production. Additionally, they play essential roles in starch processing, animal feed production, grain alcohol fermentation, malting and brewing, fruit and vegetable juice extraction, pulp and paper, and textile industries (Sulyman et al., 2020). Despite their versatile uses, a major hindrance to the widespread utilization of cellulases in industries is the high production cost of these

enzymes. To address this issue, this research work focuses on exploring the use of agrowastes, including rice straw, wheat straw, barley straw, saw dust and wild grass, *Saccharum spontaneum* that is abundant in the tropical and sub-tropical region of Nepal growing at an upper limit of 1800m, as a cost-effective and alternative source for cellulase production. By tapping into this resource, the current thesis work focuses on the isolation, screening, and optimization of cellulase producing bacteria isolated from different environmental samples using lignocellulosic biomass. The ultimate goal is to identify bacterial strains with high cellulase activity and optimize their production for the efficient conversion of Lignocellulosic biomass into bioethanol. This research contributes to the ongoing efforts to develop sustainable and environmentally friendly alternatives to fossil fuels, paving the way for a greener and more energy-efficient future (Tse et al., 2021) .

1.2 Current Studies

Over the last two decades, there has been significant research on various lignocellulosic biomass (LCB) sources as potential feedstocks for biofuel production. These sources include wheat straw, rice straw, sugarcane bagasse, barley, timothy grass, woody raw materials, forest residues, softwoods, and paper pulps (Baruah et al., 2018).

According to Davis et al (2012), using perennial cellulosic feedstocks like switchgrass and miscanthus in the United States can lead to significantly higher bioethanol production (+82%) and reduced greenhouse gas emission (-29% to-473%) compared to corn ethanol. Additionally, the utilization of municipal solid wastes as feedstocks for biofuels had multiple benefits. It not only reduces the volume of waste buried in landfills but also has the potential to meet a considerable portion of the global energy demand (Ram, Kumar,& Rani, 2021) For example, in the United States, burning 25 million tons of municipal solid waste generated about 13 billion kilowatt-hours of electriciy in 2019, reducing waste volume significantly. Similarly, in Kaohsiung, Taiwan, approximately 5400 metric tons of municipal solid waste were used to generate 135 megawatts/day of electricity (Tsai, 2019).

Cellulase, an enzyme responsible for the hydrolysis of cellulose into fermentable sugars, plays a crucial role in the bioconversion of lignocellulosic biomass. Extensive research has been conducted on cellulase-producing microorganisms, revealing the potential of bacteria as a rich source of cellulolytic enzymes. Several studies have reported the isolation of cellulase-producing bacteria from various environmental niches, such as soil, compost, and plant residues. These studies have highlighted the diversity of microbial communites and their enzymatic capabilities, providing valuable insights into the enzymatic degradation of lignocellulosic biomass. However, despite the progress made in this field, there still exists a vast untapped reservoir of cellulase-producing bacteria with unique enzymatic properties, which could significantly contribute to improving bioethanol production efficiency.

1.3 Rationale

Nepal, like many developing nations, faces the challenge of meeting its growing energy demands sustainably. The country heavily relies on imported fossil fuels for its energy

needs, leading to economic vulnerability and environmental degradation. To address this issue, the thesis topic aims to explore a potential renewable energy source, bioethanol, which can be produced from lignocellulosic biomass using cellulase-producing bacteria. Nepal is rich in lignocellulosic biomass resources such as agricultural residues, forest waste, and other organic materials, making it abundantly available for bioethanol production. Utilizing these abundant resources for bioethanol production could contribute significantly to the country's energy security while promoting sustainable waste management practices.

In addition to the energy benefits, the research will focus on isolating cellulase-producing bacteria from different environmental samples, contributing to the understanding and conservation of Nepal's unique microbial diversity. This aligns with the country's commitment to biodiversity conservation, as Nepal is renowned for its rich biodiversity in both natural ecosystems and microbial diversity. Moreover, implementing bioethanol production from locally available resources can create new opportunities for rural communities. It may open doors for entrepreneurship, generate income, and promote rural development, ultimately contributing to poverty reduction and improved living standards.

The production and utilization of bioethanol as a clean and renewable energy source can significantly reduce greenhouse gas emissions and mitigate climate change impacts. By emphasizing sustainable practices in bioethanol production, the research aligns with Nepal commitment to environmental protection and its international climate agreements. In conclusion, exploring bioethanol as a renewable energy source in Nepal has the potential to address multiple challenges simultaneously, including energy security, biodiversity conservation, socioeconomic development, and environmental sustainability.

1.4 Research Hypothesis

Bacteria isolated from diverse environmental niches possess varying cellulolytic capabilities, and through proper screening and optimization, potent cellulase-producing strains can be identified, leading to improved lignocellulosic biomass conversion and increased bioethanol production efficiency.

Null Hypothesis (H_o)

There is no significant production of bioethanol by cellulase-producing bacterial strain after its optimization when utilizing lignocellulosic biomass as substrate.

Alternative Hypothesis (H_1)

There is significant production of bioethanol by cellulase-producing bacterial strain after its optimization when utilizing lignocellulosic biomass as substrate.

1.5 Research Objectives

1.5.1 General Objectives:

To explore and harness the potential of cellulase-production on different lignocellulosic biomass waste media using bacteria screened from various environmental samples and to investigate their suitability for bioethanol production.

1.5.2 Specific Objectives:

- Identify efficient cellulase-producing bacterial strains from diverse isolates through screening and selection.
- Identification of efficient cellulase-producing isolates.
- Optimize cellulase production using different lignocellulosic biomass substrates.
- Purify crude cellulase enzyme using various purification techniques
- Evaluate the cellulase's potential for bioethanol production.

1.6 Research Scope

The world's focus on sustainable clean energy and addressing climate change has led to increased interest in lignocellulosic bioethanol as an alternative transportation fuel. With petroleum reserves, depleting and its negative impacts becoming evident, ethanol is gaining significance as a viable fuel option. Countries like India have recognized this potential and are implementing policies to promote bioethanol production from lignocellulose. India aims to produce one million liters of bioethanol annually and achieve a 20% ethanol blending in petroleum by 2030. Other countries like the USA and Brazil have already made substantial progress in ethanol production. Given the escalating air pollution, reducing dependence on petroleum should be a top priority for our landlocked nation. Adopting lignocellulosic ethanol production presents a valuable opportunity to address these challenges on a national level. To facilitate this transition effectively, this research aims to introduce a cost-effective strategy for improving the production process of lignocellulosic ethanol. Implementing this approach in the industry holds the potential to contribute significantly to the sustainable production of bioethanol.

CHAPTER 2

LITERATURE REVIEW

2.1 Structure of Lignocellulosic Biomass and its Components

Lignocellulosic Biomass (LCB) primarily consists of three main polymers: Cellulose, hemicellulose, and lignin, alongside smaller amounts of other compounds like proteins, ash, and pectin (Akthar et al., 2016). The typical composition of LCB ranges from 30% to 60% cellulose, 20% to 40% hemicellulose, and 15% to 25% lignin, although these proportions may vary depending on the source (Dahadha et al., 2017). These polymers have varying proportions depending on the source of the LCB (as shown in Table 1).

Cellulose, a key structural component of LCB, is a linear polysaccharide made up of D-glucose subunits lined by β-(1-4)-glycosidic bonds (Kumar et al., 2017). It is insoluble in water except at very low or high pH levels but can dissolve in certain solvents line ionic liquids and N-methylmorpholine-N-oxide. Cellulose offers several advantageous properties such as biocompatibility, stereoregularity, hydrophilicity, and reactive hydroxyl groups, making it a versatile resource for various applications like fibers, films, composites, as well as fuels and chemicals (Jedvert and Heinze, 2017).

Hemicellulose, the second major component of LCB, consists of short chains of various polysaccharides, including xylan, galactomannan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan, held together by β-(1,4)-and/or β-(1-3)-glycosidic bonds (Zhou S et al., 2017). Unlike cellulose, hemicellulose is easily degradable into monosaccharides due to its lower degree of polymerization and non-crystalline nature, making it widely used in industrial applications such as drug carriers, hydrogels, and cosmetics (Farhat et al., 2017).

Lignin acts as a protective barrier by covalently linking to cellulose and hemicellulose, enhancing the recalcitrance of lignocellulose. It is a complex, three-dimensional cross-linked polymer comprising phenyl propane structural units. The composition of lignin varies depending on the substitution of the methoxyl groups presents in the aromatic rings, and its units consists of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S). Lignin forms aryl ethyl linkages, such as β-O-4 and α-O-4, as well as carbon-carbon bonds like 5-5 and β- β. In contrast to cellulose or hemicellulose, this polymer lacks chains comprising repetitive subunits, which makes it very challenging for enzymatic hydrolysis (Malherbe et al., 2002).

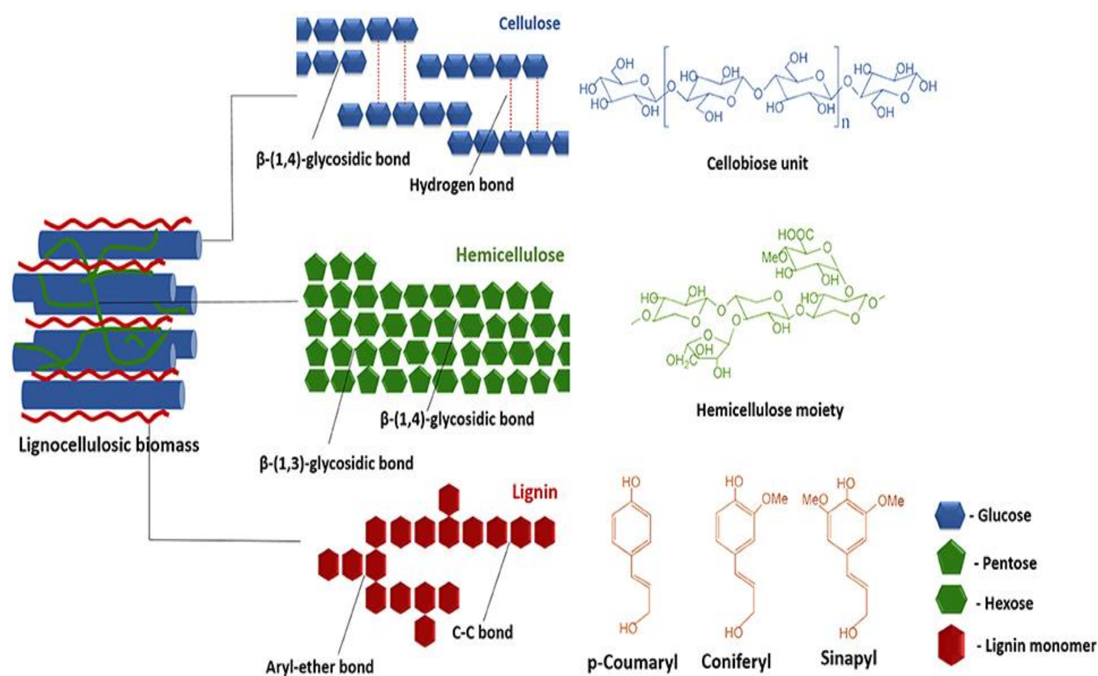


Figure 3: Structure of lignocellulose showing its various components (Baruah et al., 2018)

Table 1 Different lignocellulosic biomass and their composition (Baruah et al., 2018)

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Sugarcane bagasse	40–45	30–35	20–30	Cardona et al., 2010
Sweet sorghum bagasse	45	27	21	Kim and Day, 2011
Wheat straw	33–40	20–25	15–20	Talebnia et al., 2010
Rice straw	38	32	12	Lu and Hsieh, 2012
Rice Husk	37.1	29.4	24.1	Kalita et al., 2015b
Barley straw	38	35	16	Sun et al., 2005
Soybean straw	34	16	22	Wan et al., 2011
Corn stover	38	23	20	Wan and Li, 2010
Corn cob	41	31	12	Chen et al., 2010
Poplar	44	20	29	Kim et al., 2009
Pine	42	21	30	Sannigrahi et al., 2010
Gmelina arborea saw dust	23	-	23.3	Kalita et al., 2015a
Salvadora oleoides saw dust	24	-	21.8	Kalita et al., 2015a
Switchgrass	31	24	18	Lee et al., 2009
Coastal Bermuda Grass	30	29	23	Lee et al., 2009
Napier Grass	47	31	22	Reddy et al., 2018
Elephant Grass	36	24	28	Scholl et al., 2015
Bamboo	45	24	20	Li et al., 2015
Waste papers	65	13	1	Chen et al., 2004

2.1.1 Lignocellulosic Resources of Nepal

Lignocellulosic biomass, also known as lignocellulose, represents the vast majority of plant dry matter found on Earth stands as the most abundant source of renewable raw material (Arefin et al., 2021). Nepal possesses abundant agricultural and forest resources that

generate substantial annual quantities of waste, including rice straw, wheat straw, sugarcane bagasse, corn stover, and woody materials. These residues can be utilized as lignocellulosic biomass for energy production and other applications. Additionally, *Ipomoea cornea*, known locally as Ajamari, *Phragmites karka*, known locally as Narkat, *Saccharum spontaneum,* known locally as Kans, and *Zea mays* (corn) cobs are valuable sources of lignocellulosic biomass. These plants are abundant weeds found in Nepal's hilly and plain regions and can be easily harvested. *I. cornea* shows rapid growth and has the capacity to absorb heavy metals like Cd and Hg, making it suitable for bioremediation of polluted soils (Joshi et al., 2018)].

Saccharum spontaneum, commonly referred to as Kans grass, is a type of switch grass cultivar with promising potential as a novel substrate suitable for cultivation on wasteland (Kataria et al., 2011). It is widely distributed in tropical and subtropical regions of Asia, originating in India, and grows rapidly, providing high forage yield and seed production. The Nepali variety of *S. spontaneum* is identified by the taxonomic ID 1307 (Joshi et al., 2018

2.2 Cellulose

Cellulose, the most abundant molecule on Earth, constitutes about 50% of the total mass of organic matter and serves as a crucial structural component in plant cell walls (Lingouangou et al., 2022). It exists in either amorphous or crystalline forms, with cellulose molecules being lengthy, straight, and linear homo-polysaccharides made up of β-D-glucopyranose units connected by β-(1,4)-glycosidic bonds. The presence of reactive hydroxyl groups fosters extensive hydrogen bonding, leading to a highly organized crystalline structure and the formation of micro-fibrils in plants (Broda et al., 2022).

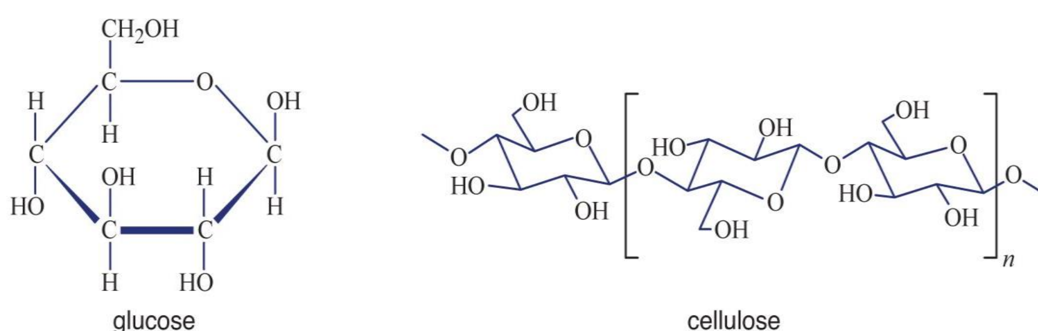


Figure 4: Structure of cellulose consisting of glucose molecule linked end to end (Source: Encyclopedia Britannica, Inc, 23 Jul. 2023)

Cellulose is insoluble in water and highly resistant to de-polymerization due to its explicit fibrous and crystalline structure, which consists of well-organized regions interspersed with less structure amorphous regions (Broda et al., 2022). This unique structure has made cellulose a target for bioconversion into valuable products such as ethanol, HMF, levulinic acid, butanol, alkanes, hexane, succinic acid, and other chemicals (Ma et al.,

2020). The first step in the conversion process is the hydrolysis of cellulose into glucose, paving the way for various bio- or chemical processes to occur (Ma et al., 2020). For successful bioconversion, factors like the nature of cellulose, cellulolytic enzyme sources, and optimal conditions for catalytic activity and enzyme production play crucial roles (Irfan et al., 2012). Researchers have been isolating and characterizing cellulose-degrading bacteria from various sources to obtain more efficient cellulases, with ongoing efforts focusing on isolating microorganisms with higher cellulase activity (Irfan et al., 2012).

2.3 Cellulase classification & its mode of action

Cellulases are enzymes belonging to the glycoside hydrolase families, responsible for breaking down cellulose into fermentable sugars. Cellulose, being highly bonded and resistant to degradation, requires multiple enzyme systems for efficient breakdown (Sharada et al., 2013). These enzymes can be derived from microorganisms cultivated on inexpensive agro-industrial materials. As cellulose is abundant and renewable, its conversion to glucose and other sugars for ethanol production is a valuable bioconversion process. The hydrolysis of cellulose into glucose involves three main types of cellulases: endo-1, 4-β-D-glucanase (endoglucanases), Exo-β-1, 4-glucanase (exoglucanases or cellebiohydrolases), and β-1, 4-glucosidase (β-Glucosidases) (Bayer et al., 1994; Singh, 1999). These enzymes work together to cleave the glycosidic bonds and transform cellulose into a usable energy source, glucose (Nagah et al., 2016).

2.3.1 Endoglucanases (EC 3. 2. 1. 4)

Endo- β - (1, 4)-glucanase, also known as endoglucanase (EC 3.2.1.4), is a crucial enzyme involved in cellulose hydrolysis. Its main characteristic is the random cleavage of β-(1, 4)-glucosidic linkages within the cellulose polysaccharide chain. This random hydrolysis generates oligosaccharides of varying lengths and creates new chain ends, which subsequently increases the specific surface area of the substrate for exoglucanase activity. As a result, endoglucanase action significantly enhances the accessibility of cellulose chain ends and reduces the viscosity of the cellulose solution (Siddiqui et al., 1999; Lynd et al., 2002).

2.3.2 Exoglucanases (EC 3. 2. 1. 91)

Exo-B-(1-4)-glucanase, also known as 1, 4- β -D-glucan cellobiohydrolases (EC 3.2.1.91), are enzymes that play a crucial role in cellulose degradation. These enzymes act by breaking down cellulose molecules, specifically cleaving cellobiose units from the non-reducing ends of the polysaccharide chains. The hydrolysis carried out by these enzymes leads to the release of either glucose, known as glucanohydrolases, or cellobiose as the primary products. Their ability to cleave cellobiose units from cellulose chains aids in the

generation of simple sugars that can be further converted into bioethanol or other valuable products (Lee et al., 2002).

2.3.3 Glucosidases (EC 3. 2. 2. 1)

Beta-glucosidase (cellobiase), as a type of cellulase, hydrolyzes the terminal beta-1, 4-glycosidic bonds of cellulose, releasing individual glucose molecules. Beta-glucosidases are particularly important because they complete the cellulose degradation process, converting cellobiose (a disaccharide unit of cellulose) into glucose, which can be further utilized by the organism for energy production decreases (Lynd et al., 2002). Cellobiases exhibit activity on various glucose β-dimers. However, as the substrate's polymerization degree increases, the hydrolysis rate of Cellobiose significantly decrease (Arantes et al., 2011).

2.3.4 Mode of Action of Cellulase

Cellulases are crucial for breaking down cellulosic substrates, but their effectiveness is hindered by the tightly packed, insoluble micro-fibrils of cellulose encased in hemicellulose and lignin. To enhance enzymatic saccharification, cellulose chains' accessibility must be improved by amorphogenesis (Figure 7, A), a process involving fiber swelling and fragmentation, leading to the release of reducing sugars (Arantes & Saddler, 2010). Cellulose, an insoluble polymer of glucose residues linked in a β-(1-4) fashion, exhibits diverse molecular architecture based on its source, such as the cell wall layer or plant type (Andersen, 2007).

The proposed mechanism for cellulose saccharification suggests that cellulases adsorb onto the surface of insoluble cellulose, causing structural loosening of the inaccessible bulk substrate. This increases the molecular disorder of tightly packed regions, exposing buried cellulose chains within the micro-fibrils while leaving them nearly unchanged on a molecular level (Coughlan, 1985) as shown in Figure7 (A). Once the cellulose network becomes accessible to the enzymes, endo- and exo-glucanases work synergistically to fragment accessible molecules into soluble cello-oligosaccharides (cellulosic molecules with a degree of polymerization of <6 units. Most of these cello-oligosaccharides are quickly hydrolyzed, with cellobiose being the primary product of cellulose hydrolysis in most native cellulase systems. Commercial cellulase systems often add an external source of β -glucosidase to fully hydrolyze cellobiose into glucose, reducing end-product inhibition and enhancing the overall reaction (Arantes & Saddler, 2010).

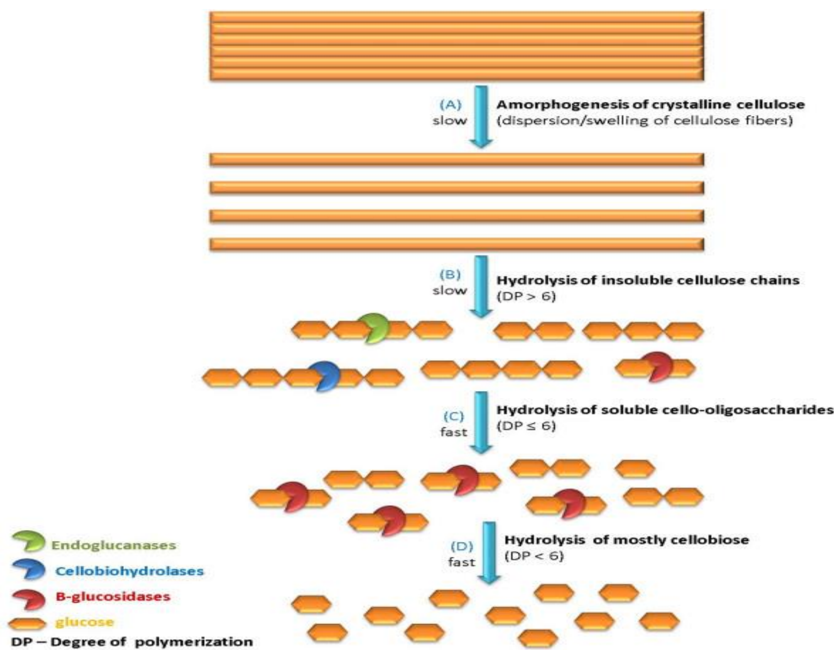


Figure 5: Proposed mechanism of mode of action of cellulases for cellulose hydrolysis (source: Arantes and Saddler, 2010)

2.4 Sources and application of Cellulases

Cellulose, a prominent component in many natural sources, can be broken down into fermentable sugar by an enzyme called cellulase, which is produced by various microorganisms like bacteria and fungi. These cellulases play a crucial role in the de-polymerization of cellulose and are inducible bioactive compounds formed by microorganisms during their growth on cellulosic matter .Cellulose-degrading microorganisms contribute to a significant material flow in the biosphere through acid or enzymatic hydrolysis, but there are still underutilized cellulose-containing raw materials and waste products (Patagundi et al., 2014).

Cellulases find extensive applications in various industries such as: textiles, food and feed, pulp and paper, laundry and detergent, agricultural and pharmaceuticals, with increasing demand for stable, highly active, and specific enzymes. Bacteria, being more efficient catalysts and genetically engineerable, hold the potential for cellulase production, with species like *Cellulomonas, Pseudomonas, Bacillus*, and *Micrococcus* exhibiting cellulolytic properties (Ariffin et al., 2006). Among bacteria, *Bacillus* species are known for their ability to produce various extracellular enzymes, such as amylases, proteinases, and polysaccharide hydrolases. Cellulase has been found to be predominantly secreted by various bacterial species, including Bacillus cereus, Bacillus licheniformis, Paenibacillus, Bacillus subtilis, and Bacillus polymyxa (Han et al., 1995). Notably, Bacillus species stand out due to their ease of genetic manipulation, setting them apart from fungi in this regard. Furthermore, these organisms have been deemed non-pathogenic, holding the Generally Recognized as Safe (GRAS) status for both animals and plants (Lam et al., 1998). Some of the industrial applications of cellulases are presented in Table 2

Table 2: Industrial applications of microbial cellulases (Source: Singh et al., 2016)

Industry	Applications
Biofuel	Production of ethanol, solvents and organic acids; production of energy-rich animal feed and with improved nutritional value
Food/animal feed processing	Improved yield and extraction of fruit and vegetables juices, clarification of fruit juices, improved maceration, color extraction of fruit and vegetables
Textiles	Biopolishing, biostoning, biofinishing
Paper and pulp	Co-additive in pulp bleaching, improved draining, de-inking
Agriculture	Control of plant pathogens and disease, improved soil quality
Medical	Antibiofilm agent, treatment of phytobezoar
others	Reduction in biomass waste, extraction of olive oil and carotenoids

2.5 Factors Affecting Cellulase Production

Organisms vary in their capacity to produce cellulase under similar conditions. The synthesis of enzymes is tightly regulated by microorganisms. As a result, to enhance their productivity, these regulatory mechanisms can be harnessed and adjusted. The yield of cellulase production seems to be influenced by a diverse set of factors, including pH, temperature, incubation period, metal ions and carbon and nitrogen sources (Zheng et al, 2018). Carbon, in particular, has a significant impact on enzyme yield, necessitating the selection of a suitable and cost-effective carbon source, along with appropriate nutrient media, culture conditions, and optimization techniques to achieve maximum endoglucanase production (Singh et al., 2016). Studies have explored the optimization of physical and chemical conditions for cellulase production in bacterial and fungal strains. For instance, Lugani et al. conducted experiments to fine-tune the chemical requirements, such as carbon source and carbon concentration, nitrogen, and nitrogen concentration, along with physical conditions like temperature, pH, and incubation time, for efficient CMCase and FPase activity from newly isolate *Bacillus sp*. Y3. Similarly, in the study of Das et al. thermophilic *Bacillus sp.* isolated from cow-dung, different parameters, including temperature, pH, inoculum size, effect of various substrates, nitrogen, incubation time, and inoculum size were carefully optimized to maximize cellulase enzyme production by thermophilic Bacillus sp. isolated from cow-dung (Lee et al., 2008).

Furthermore, Sethi et al investigated the effects of various factors such as temperature, pH, incubation time, different carbon sources in varying concentrations, and nitrogen sources on the maximum production of CMCases from various bacteria including *E. coli, Pseudomonas, Bacillus* and *Serratia.* Therefore, the optimization of physical and chemical factors plays a crucial role in improving cellulase productivity, with researchers employing diverse strategies to enhance industrial enzyme production efficiently and cost-effectively (Sher et al., 2021).

2.6 Purification and characterization of cellulase

Enzyme purification is a crucial process that involves isolating specific enzymes from complex mixtures or crude extracts. This procedure aims to obtain the highest enzyme activity possible while ensuring optimal recovery rates. To achieve successful enzyme purification, selecting the most appropriate method during the treatment stage is of utmost importance (Sher et al., 2021)

The isolation and purification of enzymes serve the purpose of distinguishing them from the other components present in cells or crude sources. The isolation of enzymes can be .accomplished through several straightforward steps, such as cell disruption, supernatant removal, and centrifugation. Cell disruption methods, including osmolysis, freeze-thaw cycles, ultra-sonication, detergent lysis, enzymatic lysis, or homogenization, are employed to break open cells and release their contents. Following this, the supernatant, which contains the desired enzymes, can be obtained using the widely known decantation process. Centrifugation, performed at specific speeds depending on the source material, is then employed based on their densities. Once isolated, enzyme purification can be achieved through five key steps (Thomas, 2019):

2.6.1 Salt precipitation using $(NH4)_2SO_4$

Ammonium sulfate is added to the enzyme solution, causing selective precipitation of proteins, including the enzyme. By adjusting the concentration of (NH4)2SO4, specific enzyme fractions can be precipitated and separated from contaminants (Duong et al., 2014)

2.6.2 Dialysis

Dialysis is a method used to remove small molecular weight impurities, salts, or buffer components from a protein solution. It involves placing the enzyme solution into a dialysis bag or dialysis tubing with a specific molecular weight cutoff, allowing the small molecules like salts and contaminants to pass through while retaining the larger enzyme molecules. By exchanging the solution several times, unwanted substances are gradually removed (Wijayanti et al., 2020).

2.6.3 Ion exchange chromatography

Ion exchange chromatography is a technique used to separate proteins based on their surface ionic charges. It involves using resin that has been modified with either positively or negatively charged chemical groups. The enzyme solution is passed through a column containing this ion exchange resin, where the enzyme binds to the resin while other molecules pass through. The purification of the enzyme is achieved by adjusting the pH or ionic strength to elute it from the column (Cseke et al., 2004)

2.6.4 Size-exclusion chromatography

Size-exclusion chromatography, commonly referred to as gel filtration or gel permeation, effectively separates proteins based on their sizes. This method relies on a chromatography column packed with a cross-linked polymer matrix. Larger proteins cannot enter the pores of the polymer and, as a result, move more rapidly through the column. On the other hand, smaller proteins can access the pores, leading to a longer and less direct path of travel within the chromatography column (Tan & Yiap, 2009).

2.6.5 Affinity chromatography

Affinity chromatography relies on a selective interaction between the target protein and the solid phase, facilitating its separation from impurities. The process shares similar steps with ion exchange chromatography [38]. This technique allows the purification of a protein based on its unique biological function or specific chemical structure [41]. When proteins exhibit a strong attraction to particular chemical groups or ligands, they covalently attach and bind to the column matrix, while other proteins flow through the column, thereby enabling effective separation from contaminants (Amersham, 2002).

Various researchers have employed a diverse range of purification techniques for enzymes. These purification methods have been carefully selected based on the unique properties and requirements of the enzymes and organisms in question. In one study conducted by Sulyman et al., 2020, the endoglucanase from *Aspergillus niger* was purified using Dialysis and Sepharose G-100 chromatographic column. In another investigation, Gaur and Tiwari purified the cellulase enzyme produced by the *Bacillus sp*. was purified by (NH4)2SO4 precipitation, dialysis, Ion exchange and gel filtration chromatography, with overall recovery of 28.8%. They first used 80% ammonium sulfate saturation, followed by dialysis, Ion exchange chromatography and subsequently purified the enzyme by gel filtration chromatography using a Sephadex G-75 column. Moreover, in a study conducted by Islam and Roy (2018), the enzyme produced by *Paenibacillus* sp. was selected for enzyme purification by ammonium sulfate precipitation, DEAE-cellulose,and CM-cellulose column chromatography, respectively. The specific activity, recovery and purification fold of the enzyme were significantly enhanced after purification and were obtained as 2655 U/mg, 35.7% and 9.7, respectively.

2.7 SDS-PAGE for molecular weight determination

SDS polyacrylamide gel electrophoresis is a powerful technique used to separate proteins based on their molecular weight. It relies on the anionic detergent SDS, which dissociates proteins into individual polypeptide subunits and imparts a uniform negative charge to each denatured polypeptide. This equal charge: mass ratio forces polypeptides to extend, eliminating shape-related effects and making chain length the primary factor governing protein migration rate during electrophoresis. By applying an electric field to denatured polypeptides loaded at the cathode end of a polyacrylamide gel, clear protein bands are obtained, arranged in decreasing order of molecular mass from cathode to anode. As a result, the migration of proteins in the gel is primarily determined by their molecular weight. The rate of movement depends on the gel's pore size and the strength of the electric field (Nowakowski et al., 2014).

Various research studies have utilized SDS techniques to determine the molecular weight of purified cellulase. For instance, Endo et al. (2001) reported that cellulase's molecular mass falls within the range of 31 to 94 kDa. In another study, *B. pumilus* was found to contain CMCase proteins with a molecular weight ranging from 30 to 65 kDa (Ariffin et al.,

2006). Furthermore, Li et al. (2009) identified putative cellulases, Cel 15 and Cel73, from *Bacillus subtilis,* which exhibited protein band sizes of 54 kDa and 27 kDa, respectively.

2.8 Determination of Cellulase activity, Protein concentration, Specific activity and purification fold

Cellulase is an enzyme that plays a crucial role in the degradation of cellulose, a major component of plant cell walls. It catalyzes the hydrolysis of cellulose into glucose units, which can be utilized as an energy source by certain microorganisms. Cellulases have numerous industrial applications, including biofuel production, textile processing, and food processing (Sonia et al., 2013)

1. Cellulase Activity:

Cellulase activity refers to the ability of the enzyme to hydrolyze cellulose. It is typically measured in international units (IU) or enzyme activity units (U). One IU of cellulase activity is defined as the amount of enzyme that releases 1 µmol of glucose per minute under standard assay conditions. The assay conditions may vary depending on the source and type of cellulase being studied (Pandey, 2014).

IU/mL= mg/mLx 1000 / Incubation time x volume of enzyme x molecular weight.

Total activity- It refers to the overall amount of protein units present in a given sample. To calculate the total activity, simply multiply the activity level of the sample by its total volume. In other words, it determines the total number of active protein units in the entire sample by considering both their individual activity and the overall volume of the sample (MSUM Biochemistry, 2006)

2. Protein concentration:

Protein concentration is the amount of protein present in a given volume of the sample. It is commonly expressed in milligrams per milliliter (mg/mL) or micrograms per microliter (µg/µL). Protein concentration can be determined using various methods, such as the Bradford assay, BCA assay, Lowry assay, or UV spectrophotometry based on the Beer-Lambert law (MSUM Biochemistry, 2006).

3. Specific Activity:

Specific activity is a measure of enzyme purity and is calculated as the ratio of cellulase activity to the total amount of protein present in the sample. It provides an indication of how much of the total protein is enzymatically active. A higher specific activity suggests a purer preparation of the enzyme (MUSM Biochemistry, 2006).

Specific Activity (units/mg) = Enzyme Activity (units) / Protein Concentration (mg)

4. Purification Fold:

Purification fold is a measure of how much the enzyme has been enriched or concentrated during the purification process. It is calculated by dividing the specific activity of the purified enzyme by the specific activity of the crude enzyme extract (MSUM Biochemistry, 2006)

Purification Fold = Specific Activity of Purified Enzyme / Specific Activity of Crude Enzyme Extract

By calculating the purification fold, one can determine how effectively the purification process enriched the enzyme of interest compared to its initial concentration in the crude extract.

5. Percentage Yield:

Percentage yield is the measure of how much of the initial enzyme activity is retained after the purification process. It indicates the efficiency of the purification process in recovering active enzyme (MSUM Biochemistry, 2006)

Percentage Yield = (Cellulase Activity of Purified Enzyme / Cellulase Activity of Crude Enzyme Extract) × 100

Different organisms produce enzymes with varying enzyme activity, specific activity, yield percentage, and purification fold. These differences can be attributed to factors such as organism type, substrate source, and various environmental and nutritional conditions. In a research conducted by Islam and Roy in 2018, they investigated the purification process of an enzyme obtained from *Paenibacillus sp.* This particular enzyme exhibited remarkable characteristics, with a specific activity of 2655 U/mg, a recovery rate of 35.7%, and a purification fold of 9.7. Notably, *Paenibacillus sp*. demonstrated a remarkable potential for cellulase production, reaching a maximum rate of 0.9 µmol ml^{-1} min^{-1}. Furthermore, Sulyman's work in 2020 focused on the purification of the Cellulase enzyme derived from *Aspergillus niger.* The purification process resulted in an impressive 68.12-fold increase in enzyme concentration, while achieving a yield of 3.87% and a specific activity of 484.3 U/mg (Sher et al., 2021).

2.9 Overview of Lignocellulosic bioethanol production

Lignocellulose, the most abundant natural polymer, plays a vital role in the carbon cycle through its utilization and degradation. To increase its utilization, the material can be hydrolyzed into fermentable sugars, which are then used to produce renewable energy materials and other fermented products. Given the rapid depletion of fossil fuel reserves, there is a growing interest in using biofuels as a renewable energy source (Rashid et al., 2011). The appeal of bioethanol as a fuel lies not only in its renewability but also in its

environmentally friendly nature, offering a potential solution to issues like global warming and acid rain caused by fossil fuels (Yamada et al., 2010).

Currently, a significant portion of global bioethanol production comes from first-generation processes involving fermentation of food and feed stocks such as molasses, sugar cane, corn, and potatoes. However, this approach raises concerns about food price inflation and food security due to the demand for abundant feedstock bioethanol (Bušić et al., 2018). To address these issues, second-generation bioethanol production from lignocellulosic biomass has emerged as a promising alternative. Materials like corn stover, wheat straw, sugarcane bagasse, oat hull, rice straw, wood chips and various weeds such as Switch grass, *Saccharum spontaneum, Lantana camara, Eichhornia crassipes* (water hyacinth),etc. offer widespread abundance and cost-effective procurement (Joshi et al., 2011). The overall process involves three key steps: mechanical processing and pretreatment of the material, hydrolysis of the pretreated material, and fermentation of the hydrolyzed material by a suitable microorganism to produce ethanol (Thomas et al., 2021).

2.9.1 Pretreatment of Lignocellulosic Biomass

Pretreatment of lignocellulosic biomass is a crucial first step in their conversion to ethanol. This process involves disrupting the structure of lignocellulose by breaking down cross-linkages between its polymers, reducing cellulose crystallinity, and increasing surface area and porosity. These changes make it easier for enzymes to access carbohydrates, improving hydrolysis and fermentation yields. However, the presence of lignin poses challenges to efficient polysaccharide hydrolysis. Lignin restricts enzyme access to cellulose and hemicellulose, and it can also bind and deactivate enzymes, making its removal essential during pretreatment (Broda et al., 2022)

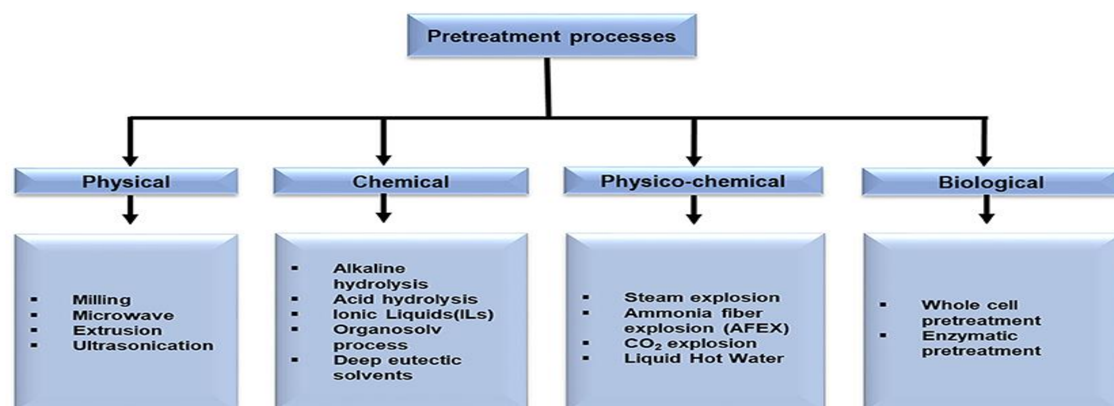


Figure 6: Flow chart diagram representing pretreatment technique (Source: Baruah et al., 2018)

These pretreatment methods fall into different categories, such as Physical, chemical, physicochemical, and biological, and often involve combinations of these approaches. Examples include milling, irradiation, microwave, steam explosion, ammonia fiber explosion (AFEX), supercritical CO2 treatment, SO2, alkaline hydrolysis, liquid hot-water pretreatment, wet oxidation, Ozonolysis, dilute and concentrated acid hydrolyses, and biological pretreatments (Baruah et al., 2018). Each of these methods has distinct advantages and disadvantages, and ongoing research and development hold great

potential for improving efficiency and lowering production costs. Integrating various biomass pretreatment methods with other processes like enzymatic saccharification, detoxification, fermentation of hydrolysates, and product recovery can significantly reduce the overall cost of using lignocellulose for practical applications. Therefore, the future success of commercial-scale lignocellulosic conversion hinges on advancements in pretreatment technologies, cellulolytic enzyme-producing microorganisms, comprehensive utilization of biomass components, and effective process integration (Isikgor & Becer, 2015).

Liquid Hot water pretreatment method:

Liquid Hot Water (LHW) pretreatment is a simple and eco-friendly technique that involves subjecting lignocellulosic biomass to high-pressure water (up to 5 MPa) at elevated temperatures (170 to 230 ◦C). This process permeates the material, leading to hemicellulose hydrolysis, partial lignin removal, and cellulose hydration, making cellulose more accessible to enzymes for further processing. One of the remarkable advantages of this method is the production of minimal inhibitory compounds. Additionally, it proves to be a cost-effective and environmentally friendly alternative, as it eliminates the need for an energy-intensive preliminary reduction in feedstock size and avoids the use of chemicals or corrosion-resistant hydrolysis reactors (Baruah et al., 2018). According to Joshi et al (2018), *S. spontaneum* biomass when pretreated with hot water at 100°C for 2 hours has releases maximum of reducing sugar in comparison with other pretreatment methods.

2.9.2 Enzymatic Hydrolysis

Pre-treatment of raw materials is followed by hydrolysis, which converts hemicellulose and cellulose into fermentable sugars. Hydrolysis can be done chemically or enzymatically. Enzymatic hydrolysis is preferred due to higher conversion efficiency, milder conditions, and the use of biodegradable and non-toxic reagents (Yang et al., 2002). Cellulose, with its crystalline structure, requires three cellulase enzymes for effective hydrolysis: Endo-1, 4-β-glucanases (EG) for random 1, 4-β-glucan bond cleavage, Exo-1, 4-β-D-glucanases for releasing D-glucose and cellobiose from 1, 4-β-glucan, and β-D-Glucosidase for converting cellobiose and other glucose oligomers into D-glucose (Lynd et al., 2002).

2.9.3 Ethanol Fermentation

After extracting sugars from cellulosic materials, they undergo fermentation with yeast or bacteria to produce ethanol. Cellulosic materials are processed to obtain sugars, mainly glucose and xylose (pentoses). The most widely used microorganism for ethanol production is *Saccharomyces cerevisiae* due to its ability to efficiently ferment various sugars (glucose, maltose, galactose, fructose, and sucrose) and produce ethanol at pH values between pH 3.5-6.0) and temperature from 28°C to 35°C (Eliasson et al., 2000). However, *S. cerevisiae* cannot ferment pentoses. Other microorganisms like *Pitchia stipitis*, *Candida shehatae*, and bacteria such as *Zygomonas mobilis* and *Clostridium sp*.

can ferment both hexoses and pentoses to ethanol (Zang et al., 1995). Developing strains capable of utilizing both types of sugars simultaneously would enhance ethanol production efficiency from lignocellulose-derived sources. Genetic engineering of *S. cerevisiae* and *Z. mobilis* is being explored to enable them to ferment pentoses and improve ethanol yield and tolerance (Goldschmidt, 2008).

3.0 Application of Bioethanol

Introduction

Bioethanol is a promising biofuel derived from renewable sources like agricultural and forestry residues, as well as organic waste. It is primarily produced by fermenting sugars from starch-rich crops. When blended with petrol, it serves as a high-octane additive and reduces harmful emissions due to more complete combustion. The key environmental advantage lies in its composition, as it is entirely derived from biological materials. When burned, bioethanol emits cleaner emissions, and the carbon dioxide released is absorbed by plants through photosynthesis, making it potentially carbon-neutral (Pirolini, 2015)

Applications

Bioethanol serves as a versatile alternative to gasoline, capable of being mixed with petrol in varying proportions. In existing petrol engines, blends of up to 15% bioethanol are commonly used. This substitution results in a higher octane number (meaning it burns more efficiently and cleanly) leading to increased thermal efficiency and improved compression ratios in engines. The applications of bioethanol extend beyond transportation fuels. It finds utility as a clean-burning fuel for power generation through thermal combustion and cogeneration systems (Pirolini, 2015). Additionally, the chemicals industry utilizes bioethanol as a vital feedstock. Moreover, it serves as a fuel for fuel cells, facilitating thermochemical reactions. Furthermore, bioethanol proves advantageous for residential use, particularly in flueless fireplaces, where it does not necessitate a chimney. Its multifaceted nature makes it a promising and environmentally friendly choice across various sectors. (Marszałek & Kamiński, 2008).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Research design

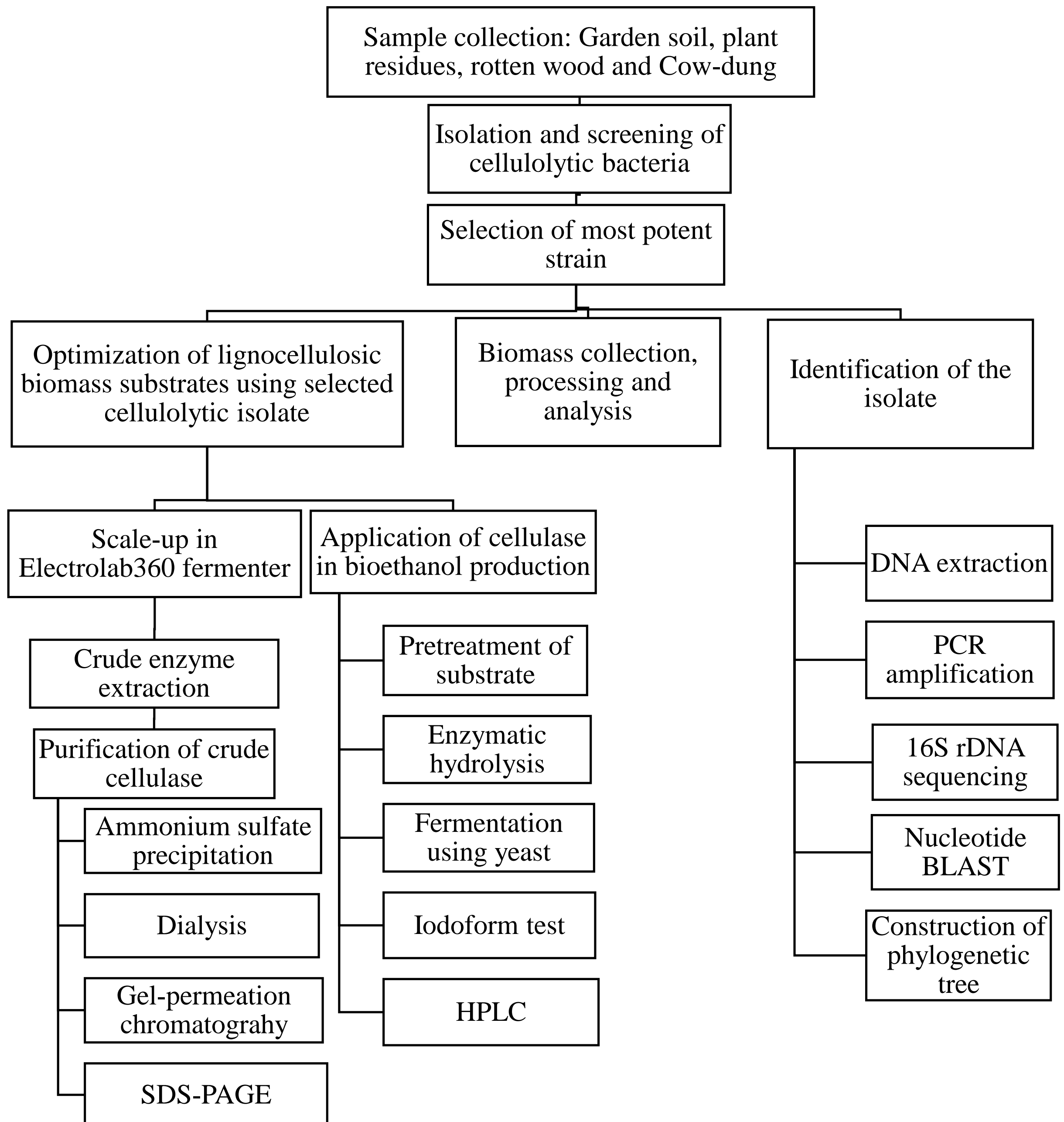


Figure 7: Flow chart of the overall process

3.2 MATERIALS

This research was conducted at the Central Department of Biotechnology, Tribhuvan University, Kirtipur, Nepal, from December 2021 to March 2023. The majority of the research involved hands-on laboratory experiments. Department provides all necessary materials, equipment, bacteriological media (Hi-Media Company), and reagents essential for the study.

3.3 METHODS

3.3.1 Collection of sample

Garden soil, cow dung, plant residues, and rotten wood were collected as sample sources from different areas of Kathmandu i.e Lainchour, Dhapasi, Chovar, and Kritipur for the isolation of cellulase-producing microorganisms. And all these samples were collected in sterile plastic bags, and transported under aseptic conditions to the laboratory for further investigations.

3.3.2 Isolation of bacteria

Cellulolytic bacteria were isolated using a serial dilution method. A 1g sample was mixed with 10 mL autoclaved distilled water and activated in a 30°C incubator for 1-2 hours. Dilutions up to 10-7 were created. Bacterial samples (100 µL) from each dilution were spread on cellulolytic specific CMC agar media (K_2HPO_4 0.2g/L, KH_2PO_4 0.2g/L, $MgSO_4$ 0.2 g/L, NaCl 0.2 g/L, $NaNO_3$ 1g/L, $CaCO_3$ 0.01 g/L, Yeast extract 0.5 g/L, CMC 10g/L , Agar 15g/L, pH adjusted to 7), and incubated at 30°C for 48 hours. Morphologically distinct colonies were selected, purified through repeated streaking on the same medium, and stored at 4°C. These pure bacterial cultures were then used to screen for cellulolytic activity.

3.4 Screening of cellulose-producing organisms

For the assessment of cellulolytic activity, two methods were employed:

3.4.1 Plate Hydrolysis Method

Primarily, Isolated bacteria were assessed for their cellulolytic activity using Cellulose Congo Red Agar media (CRAM) containing KH_2SO_4 0.5g/L, $MgSO_4$ 0.25 g/L, Cellulose 2g/L, Agar 15 g/L, Congo-Red 0.2 g/L, gelatin 2 g/L and distilled water (1L) adjusted to pH 6.8-7.2. Individual isolates were streaked onto these agar plates and incubated at 30°C for 48 hours. Cellulose degradation was indicated by the development of a clear hydrolysis zone around bacterial colonies. Colonies exhibiting Congo red discoloration were identified as positive cellulose-degrading strains and taken for secondary screening tests for confirmation (Gupta et al., 2012).

3.4.2 Well Diffusion Method

The cellulolytic activity of positive bacterial colonies obtained from the plate hydrolysis method was confirmed through additional screening using the well diffusion method. In

this step, pure colonies of above selected isolates were cultivated in a CMC culture broth (K_2HPO_4 0.2g/L, KH_2PO_4 0.2g/L, $MgSO_4$ 0.2 g/L, NaCl 0.2 g/L, $NaNO_3$ 1g/L, $CaCO_3$ 0.01 g/L, Yeast extract 0.5 g/L, CMC 10g/L, pH adjusted to 7), and incubated at 30°C for 48 hours. Following this incubation period, 1 mL of the culture suspensions were subjected to centrifugation at 5000 rpm for 5 minutes, and the resulting supernatant were used as crude enzyme.

Next, a sterile Cork borer created 5 mm diameter holes in agar plates containing 1% CMC and 1.5% Agar. Following this, 100 µL of the above prepared crude enzyme extract were placed in each hole, and then incubated at 30°C for 48 hours. After incubation, in order to visualize the cellulose hydrolysis zone, a 0.1% Congo red solution was applied and left for 10-15 minutes. After de-staining the dye and washing the plate with 1M NaCl, the hydrolysis zone's diameter was measured against a dark red background. Positive isolates were selected for further analysis based on hydrolysis zone size (Sharma et al., 2015)

3.4.2.1 Analysis of reducing sugar for quantification of Cellulase

After identifying promising cellulolytic isolates through qualitative screening, the positive isolates were subjected to quantitative analysis using the CMCase assay method outlined by Miller in 1959. CMCase activity was determined by incubating 0.5 mL of crude enzyme (prepared as same way as in 3.4.2) mixed with 1 mL of substrate (1.0% CMC in 0.05 M Sodium citrate buffer, pH 4.8) and 1 mL of sodium citrate buffer at 55°C for 30 minutes. The reaction was stopped by adding 3.0 mL of DNS reagent and boiling the tubes in a water bath at 100°C for 5-10 minutes. Absorbance at 540 nm was recorded against the blank, and glucose standards were used to determine the amount of reducing sugars formed. A glucose standard curve drawn for estimating reducing sugar is shown in Appendix II. One unit of CMCase activity was defined as the amount of enzyme that released 1 µmole of glucose per minute (Miller, 1959).

3.4.2.2 Glycerol stock preparation

Glycerol stocks of the selected cellulolytic bacterial strains were prepared by adding 500 µl of overnight bacterial culture to 500 µl of 50% sterile glycerol in a micro-centrifuge tube and mixing well. And the tubes were incubated by gradually cooling from 4 ^{O}C to -20 ^{O}C.

3.5 Identification of microorganisms.

The most effective cellulolytic bacteria were chosen and then underwent various morphological and biochemical assessments to determine their identification.

3.5.1 Colony morphology

Bacterial colony morphology was studied using a Nutrient Agar (NA) medium. The study involved observing and describing the color, shape, size, margin, elevation, and opacity of the bacterial colonies. To conduct the analysis, a fresh 24-hour bacterial culture grown on NA was utilized.

3.5.2 Microscopic study

Gram staining was performed on an overnight bacterial culture grown on Nutrient agar to distinguish between gram-positive and gram-negative bacteria and determine their structure and shape. A bacterial smear was prepared on a slide using a loopful of the culture. The smear was sequentially treated with crystal violet for 30 seconds, Gram's iodine for 10 seconds, and then decolorized with alcohol. After being washed and dried, the smear was stained with Safranin for 30 seconds, washed, and gently dried with absorbent paper. Finally, the slide was examined under a microscope for analysis.

3.5.3 Biochemical test of bacteria

Bacterial isolates were presumptively identified through a series of biochemical tests. These tests included assessing endospore formation, catalase production, Methyl Red Voges Proskauer (MRVP) reaction, indole production, citrate utilization, Sulphur Indole Motility (SIM) test, Triple Sugar Iron Agar (TSIA) test, urease activity, and oxidative/fermentative traits. The bacteria were cultured in specific media within test tubes and then incubated overnight at 30°C. Color changes were observed in tests for citrate utilization, urease activity, MRVP reaction, and TSI reactions, which also included Hydrogen Sulphide (H_2S) production. The SIM test was used to determine H_2S production and motility.

3.5.4 Molecular identification of the selected strain and phylogenetic analysis

3.5.4.1 Genomic DNA Extraction

A modified CTAB-based DNA extraction method by William et al., (2004) was employed to extract DNA from cellulolytic isolate U1C4. The bacterial culture in Nutrient broth was centrifuged at 8000 rpm for 5 minutes, and the supernatant was discarded. Cells were re-suspended in 570 µL of TE buffer, followed by gentle mixing with approximately 10 µg of lysozyme. The mixture was then incubated at 37°C for 10 to 60 minutes. Next, 6 µL of proteinase K (10mg/mL) and 30 µL of 20% SDS were added, and the suspension was further incubated at 37°C until it became clear and viscous. Following this, 100 µL of 5M NaCl was added, thoroughly mixed, and the suspension was subjected to a 65°C water bath for 10 minutes. A preheated solution of 80 µL of 1M CTAB/1.5M NaCl was introduced and mixed well, followed by another 10-minute incubation at 65°C. The supernatant obtained after centrifugation at 6000 rpm for 10 minutes at room temperature was carefully collected and combined. An equal volume of chloroform-isoamyl alcohol (24:1, v/v) was added, and the aqueous phase was separated by centrifugation at 10000 rpm for 5 minutes. This extraction process was repeated at 15,000 rpm using phenol-chloroform-isoamyl alcohol. The aqueous phase was isolated again, and after another extraction with chloroform-isoamyl alcohol, it was precipitated with 0.7 volume of isopropanol at room temperature for 1 hour. The resulting nucleic acid pellet was obtained by centrifugation at 12,000 rpm for 20 minutes at room temperature, washed with 70% ethanol, air-dried, and then re-suspended in approximately 50 µL of Tris EDTA (TE) buffer (William et al., 2004). Verification of genomic DNA was performed through 1% agarose gel electrophoresis, and quantification was accomplished using a Nano-drop

spectrophotometer to determine concentration and purity. Any remaining DNA was stored at -20°C for future use.

3.5.4.2 PCR Amplification of Genomic DNA

The bacterial DNA was subjected to amplification of the 16S rDNA region utilizing a pair of universal primers: 16sBakt341F having sequence CCT ACG GGN GGC WGC AG(Size 17bp) as the forward primer and 16sBakt805R having sequence GA CTA CHV GGG TAT CTA ATC C (Size 21bp) as the reverse primer (Illumina, 2013) .

Table 3: Components of PCR used for amplification of genomic DNA

Components	Volume(µL)
2X Kappa (Master mix)	12.5
Forward primer:16sBakt341F	5
Reverse Primer:16sBakt805R	5
Template	2.5
Total	25

Table 4: PCR Condition for amplification of genomic DNA

STEPS	TEMPERATURE	TIME	CYCLES
Initial denaturation	95	3 min	1
Denaturation	98	30 s	35
Annealing	65	25 s	
Extension	72	20 s	
Final Extension	72	5 min	1
4°C		Hold	

The PCR was carried out in a Bio-rad thermal cycler using different components and conditions as mentioned above in Table 3 and 4 respectively. Thus amplified PCR products were analyzed using a UV trans-illuminator.

3.5.4.3 Sequencing and Sequence Analysis

The amplified PCR products of the isolate U1C4 were carried to CMDN (Center for Molecular Dynamics Nepal), Kathmandu, Nepal for sequencing purposes. The sequencing was carried out in both directions using forward and reverse primers in ABI310 Genetic Analyzer. The raw sequences were quality trimmed. And the sequences with both forward and reverse reads were aligned into a con-census sequence. After that sequences were subjected to nucleotide blast at Gene bank database Release 251.0 (August 26, 2022)

using blastn web v2.13.0 for the identification of an organism species. Also, a phylogenetic tree of the isolate with other related strains was constructed using the neighbor-joining method (NJ) in the MEGA11 software program (Tamura et al, 2013). The raw sequence and Electropherogram image of the sequence are given in Appendix III.

3.6 Lignocellulosic biomass collection, processing, and analysis

Various sources of renewable lignocellulosic biomass were collected during the month of August, 2022 from different locations around the Kathmandu Valley i.e Kirtipur, Chovar, Tokha, Lainchour, and Syangja district, Nepal. These sources included *Saccharum Spontaneum* (Kans grass), rice straw, wheat straw, barley straw, and wood dust. To prepare these substrates, they were initially rinsed to eliminate any impurities. Subsequently, they underwent sun drying to eliminate moisture, followed by an air-drying period of 10-15 days. The dried materials were then processed in a mixer-grinder to reduce particle size, and the resulting biomass was sieved using Brass wire mess (Gilson company, INC) to achieve finely powdered particles ranging from 250 to 500 µm. The processed biomass was meticulously collected and individually packaged in plastic bags. These packages were stored in a temperature-controlled, dry environment at room temperature for subsequent analysis.

3.6.1 Pretreatment of Biomass

The biomass was subjected to pretreatment using the hot water method. To begin with, 10 g of powdered biomass was mixed with 100 mL of distilled water in a 250 mL reagent bottle. This mixture was then placed in an iron vessel that had been preheated for approximately 30 minutes, with some water added to it. The iron vessel, along with the reagent bottle, was then kept in a hot air oven set at 120 ^{0}C for 2 hours for the treatment. After the incubation period, the iron vessel was removed from the hot air oven and placed in cold water for a while. The pretreated substrate was then filtered, and the remaining residue was spread evenly on aluminum foil and kept in a hot air oven set at 50°C for 24 hours to dry. Once the drying process was complete, the pretreated biomass was carefully packaged and sealed in a Ziplock plastic bag. Finally, it was stored in a cool, dry cabinet at room temperature until further use. This method of hot water pretreatment efficiently prepared the biomass for further processing and utilization. The detailed procedure followed ensured that the pretreated biomass was of high quality, suitable for its intended application (J. Joshi & Bhattarai, 2018).

3.7 Analysis of sugar and other compounds in biomass substrates by High-Performance Liquid Chromatography (HPLC)

Digestion of sample and generation of calibration standard

A series of solutions containing different concentrations (1mg/L, 5 mg/mL, 10 mg/mL, 15 mg/L, 20 mg/mL) of glucose as well as fructose, xylose, glycerol and ethanol was prepared in separate reagent bottles. These solutions were used to create a calibration curve. In five separate heat-resistant reagent bottles, 0.3 g each of five different biomass samples (i.e. powdered form of *Saccharum spontaneum*, rice straw, wheat straw, barley straw, and wood dust) were placed. To each reagent bottle, about 3mL of 72% H_2SO_4 (Sulphuric acid)

was added, and the bottles were placed in a water bath maintained at a temperature of 30±3 ^{O}C for 60 minutes. During this incubation period, the bottles were stirred every 10 minutes without being removed from the water bath. After incubation, the concentrated acid in the bottles was diluted from 72% to 4% by adding 84 mL of deionized water. To ensure thorough mixing and avoid separation between high and low-concentration acid layers, the reagent bottles were inverted several times. The acid-sample mixture was then autoclaved at 121°C for 1 hour and subsequently cooled to room temperature before further use (Van Wychen S. et al., 2015).

HPLC Analysis

From the above prepared digested sample, a portion of the hydrolysis liquor, measuring 20 mL, was transferred to a separate flask. To achieve a neutral pH of 6, calcium carbonate was added gradually while swirling the samples. The pH was regularly monitored during the addition of calcium carbonate. Once the desired pH was reached, the sample was allowed to settle, and the clear liquid above it was carefully poured into tubes, leaving behind any sediment. The neutralized sample was then centrifuged, and filtered using a 0.2 mm syringe filter. The filter hydrolysate was placed in a clean and labeled HPLC vial, which was tightly capped. The calibration standard and the biomass samples underwent analysis using a specialized column called Aminex HPX-87H, a carbohydrate and organic acid-specific column (300*7.8 mm). The mobile phase consisted of a mixture of HPLC water and acetonitrile, with a ratio of 25:75, and it was pumped through the column at a flow rate of 0.6 mL/min. The temperature of the column was maintained at 50°C and the sugar and other compounds present in the samples were detected using a UV 210 nm refractive index detector (RID). A 20 µL volume of the sample was injected into the system for analysis (NREL protocol, 2015)

3.8 Qualitative and Quantitative method of Cellulase activity estimation

3.8.1 Crude Enzyme Production

A pure colony of isolate U1C4 was inoculated in the culture tube containing CMC broth of following composition: K_2HPO_4 (0.2g/L), KH_2PO_4 (0.2g/L), $MgSO_4$ (0.2g/L), NaCl (0.2g/L), $NaNO_3$ (1g/L), $CaCO_3$ (0.01 g/L), Yeast extract (0.5 g/L) and CMC (10g/L). The bacteria were incubated at 30°C for 24 hours. After 24 hours of incubation, the culture broth was then centrifuged at 5000 rpm at 4 ^{O}C for 5 minutes. The supernatant thus obtained was used as a crude extracellular enzyme and stored at -20 ^{O}C for subsequent use in further experiments (Sharma et al., 2015).

3.8.2 Quantification of Cellulolytic Activity by Carboxy Methyl Cellulase Assay

The quantitative determination of cellulase activity was carried out by Carboxy Methyl Cellulase (CMCase) assay as described by Miller, (1959) based on measuring the concentration of reducing sugars produced during the enzymatic reaction. To perform this assay, a reaction mixture containing 0.5mL crude enzyme solution was added to 1 mL of substrate (1 % CMC prepared in 0.05M sodium citrate buffer, pH 4.8) plus 1 mL sodium citrate buffer (PH 4.8, 0.05 M). The reaction mixture containing 0.5 mL boiled enzyme and 0.5 mL water instead of the enzyme were used as an enzyme and substrate blank respectively. Both test and blank samples were incubated at 50 ^{O}C for 30 minutes to

perform the enzyme-substrate reaction followed by the addition of 3 mL of DNS. It was heated to boil for 10 min resulting in the formation of a colored complex and immediately cooled. After cooling, this colored complex was then quantified by measuring the absorbance at 540 nm against a blank that contained all the reagents except for crude enzyme using a UV Spectrophotometer (Thermo Scientific, U.K) The activity of the enzyme is expressed in the international unit (IU/mL or U/mL). The absorbance of the sample tubes was translated by constructing a linear glucose standard curve using absolute amounts of glucose plotted against A540nm. One unit of CMCase activity was expressed as 1 µmole of glucose liberated per mL enzyme per minute (Ghose, 1989). All experiment was performed in triplicates.

3.9 Optimization of Cellulase production using different Lignocellulosic biomass as substrates

The optimization of cellulase production using lignocellulosic biomass as a substrate is a complex process that requires careful selection of microorganisms, optimization of growth conditions, selection of appropriate biomass substrates, and scaling up of the production process. To determine the potential suitability of agro-based waste as a substrate for enzyme production, different substrate such as *Saccharum spontaneum*, rice straw, wheat straw, barley straw, and wood dust were utilized as a carbon source in the production medium:[K_2HPO_4(0.2g/L), KH_2PO_4(0.2g/L), $MgSO_4$(0.2g/L), NaCl(0.2g/L), $NaNO_3$(1g/L), $CaCO_3$(0.01 g/L), Yeast extract (0.5 g/L) and CMC (10g/L] under submerged conditions in place of Carboxymethyl cellulose(CMC), which was commonly used as a carbon source in the medium. After that, for maximum cellulase production, incubation period, temperature, pH, nitrogen source and concentration, carbon source and concentration, inoculum size, etc. were adjusted while maintaining all other factors constant. For each optimization, during the enzyme production, a control 25 mL culture tube with 10 mL sterile media without inoculum was also incubated for cross-checking along with 0.1% inoculum transferred in another culture tube as a test. For both sets of culture tubes except inoculum, all the parameters for optimization were kept constant. Also, during each optimization, the optimum parameter obtained was used for the subsequent optimization parameter.

For this, 0.1 g of each type of pretreated biomass was weighed and added to five sets of 10 mL (w/v) of the production medium (without CMC) separately in culture tubes. Next, 100 µl of an overnight culture of a selected cellulolytic isolate was added to each culture tube, and the tubes were incubated at 30 ^{O}C for 24 hours. After the incubation period, cellulase activity was assayed using the DNS method (Miller, 1959) for each set separately to determine the optimal condition of each substrate for maximum cellulase production. Every experiment was carried out three times, and the standard error of the mean was computed.

3.9.1 Effect of pH on cellulase production

In this study, the impact of pH on enzyme activity was investigated. To create different pH ranges, overnight cultured inoculums were introduced into media prepared with specific

buffers. These buffers included citrate (pH 3-5), phosphate (pH 6-7), Tris (pH 8), and glycine-NaOH (pH 9-10). The pH levels were carefully adjusted using 1N HCl and 1N NaOH solutions. The samples were then incubated for 24 hours at a temperature of 30°C. After the incubation period, the cell-free culture filtrate was collected to serve as the enzyme source. Subsequently, the DNS assay was performed for each set of samples to determine the enzyme activity under the different pH conditions.

3.9.2 Effect of temperature on cellulase production

The ideal temperature range for optimal enzyme activity varies depending on the specific enzyme and microorganism involved in the bioprocess. To determine the best incubation temperature for the selected strain U1C4, the production media containing the inoculated culture was subjected to different temperatures (ranging from 20°C to 50°C) for a duration of 24 hours. The amount of glucose produced was measured using the DNS method (Miller, 1959) for each temperature setting, aiming to identify the temperature at which maximum enzyme production occurs.

3.9.3 Effect of incubation time on cellulase production

The optimal incubation time for cellulolytic bacteria was determined by monitoring the growth rate of the bacteria over time in the production medium which contains pretreated biomass as substrate. This was done by culturing the inoculum of the selected cellulolytic isolate in the medium (pH 8) at 35 ^{0}C. The incubation was done for up to 6 days and cellulase activity was measured in every 24 hours by Carboxy methyl cellulase assay as described by Miller (1959).

3.9.4 Effect of carbon source on cellulase production

The optimization process involved evaluating the impact of various substrates, such as rice straw, wheat straw, barley straw, wood dust, *Saccharum spontaneum*, and CMC, as potential carbon sources. Each substrate was introduced into the media at a concentration of 1% (w/v) under a pH of 8. Subsequently, the culture tubes containing the diverse substrates were subjected to an incubation period of 72 hours at a temperature of 35°C – the determined optimum conditions. Following the incubation, the culture broth underwent centrifugation at 6000 rpm for 10 minutes at a temperature of 4°C. This process resulted in the collection of the supernatant, which served as the source of crude enzymes. The cellulase activity was then assessed using the CMCase method, a standard technique for measuring cellulase efficacy, to identify the most suitable substrate for the carbon source (Lugani et al. in 2015).

Also, the impact of varying concentrations (0.5%, 1%, 1.5%, 2%, 2.5%, 3% v/v) of a selected carbon source was studied to investigate its influence on cellulase production.

3.9.5 Effect of Nitrogen source on cellulase production

Different nitrogen sources, including peptone, urea, yeast extract, ammonium sulfate, and ammonium chloride, were assessed to determine their influence on enzyme production. The cellulolytic activity was measured using the CMCase assay. To investigate the effect on cellulase synthesis by *Bacillus cereus sp.* strain U1C4, the impact of varying concentrations (0.5%, 1%, 1.5%, 2%, 2.5%, 3% v/v) of a chosen nitrogen source was also examined.

3.9.6 Effect of inoculum size on cellulase production

The influence of different amounts of the bacterial strain *Bacillus cereus sp.* strain U1C4 inoculum (ranging from 0.5%, 1%, 2% to 5% v/v) was investigated about its effect on cellulase activity after a 72 hour incubation period.

3.10 Scale-up of Cellulase production to 3.5 L media in Fermenter under optimized conditions

In this experiment, the cellulase enzyme production process was scaled up from a 25 mL culture tube to a 5-liter Electrolab 360 fermenter using optimized media. A 3.5 liters production media prepared at optimum pH 8, and *Saccharum spontaneum* was chosen as the best carbon source for cellulase production among the options of rice straw, wheat straw, barley straw, and wood dust. A 350 g of finely powdered and pretreated *Saccharum spontaneum* was added to the autoclaved media and mixed thoroughly. Next, 4% inoculum i.e. 140 mL of an overnight culture isolate U1C4 was carefully poured into the fermenter under aseptic conditions. The fermenter was then sealed with a cotton plug, and the temperature and pH were adjusted to 35 ^{O}C and 8 pH, respectively, with agitation of 100 rpm. The incubation was initiated and allowed to run for 7 days, with sampling performed every 24 hours to quantify the cellulase enzyme production using the CMCase assay. Also, to observe the impact of agitation on cellulase production, the same process was repeated without agitation. Both experiments were carried out to determine the optimal conditions for cellulase production in the fermenter.

Table 5: Constituents in production media (optimized)

Constituents	For 1 L	For 3.5L
K_2HPO_4	0.2g	0.7g
KH_2PO_4	0.2g	0.7g
$MgSO_4$	0.2g	0.7g
NaCl	0.2g	0.7g
$NaNO_3$	1g	3.5g
$CaCO_3$	0.01g	0.035g
Peptone (1%)	10g	35g
Saccharum spontaneum (2%)	20g	70g

3.11 Partial Purification of Crude cellulase enzyme

Approximately 50 mL of culture broth was aseptically transferred into a clean and sterile conical flask from the Elecrolab 360 fermenter. This culture broth was a crucial component of the cellulase scale-up process carried out by U1C4. The entire process adhered to meticulously optimized conditions, including agitation at 100 rpm. Following the fermentation phase, the 50 mL culture broth underwent centrifugation at 5000 rpm and

4°C for a duration of 5 minutes. This step was aimed at effectively eliminating all cellular debris present. Consequently, the resulting supernatant thus obtained was used as a crude extracellular enzyme and then harnessed for subsequent purification procedures. The resulting crude enzyme preparation was subjected to partial purification and further analyzed, which involved purification techniques such as ammonium sulfate precipitation, dialysis, and gel filtration/ permeation chromatography.

3.11.1 Determination of protein concentration and enzymatic activity

Protein concentrations were determined by measuring the absorption at 280 nm for each partially purified enzyme obtained from the above-listed purification technique. The quantification of protein in each fraction was carried out by Bradford assay (Bradford, 1976) with Bovine Serum Albumin (BSA) used as the standard protein. It was used to find the protein content in the sample (Appendix II). The protein concentrations were expressed in milligrams per milliliter (mg/mL). Whereas, the quantitative determination of enzymatic activity was carried out by Carboxy Methyl Cellulase (CMCase) assay as described by Miller, (1959) based on measuring the concentration of reducing sugars produced during the enzymatic reaction (previously mentioned in 3.8.2).

3.11.2 Ammonium sulfate precipitation

The above-prepared crude extracellular enzymes (3.11) were used for the purification process which was carried out at a temperature of 4°C. To purify the enzyme, the crude supernatant was subjected to salt precipitation sequentially using ammonium sulfate, starting from 20% saturation and increasing up to 80% saturation. Optimization of ammonium sulfate precipitation was done according to the protocol suggested by Harris (2001). The amount of ammonium sulfate required giving 20, 40, 60, and 80% saturation can be observed in Appendix IV. A weighed amount of ammonium sulfate required was slowly added to the aliquots stirring in a magnetic stirrer at 4 ^{O}C. The mixture was allowed to stand for 12 hours, following which the precipitate was collected by centrifugation at 13000 rpm for 30 minutes at 4 ^{O}C. The resulting precipitate was dissolved in 1-2 mL of 50 mM phosphate buffer at a pH of 7. Finally, enzyme activity was measured in Congo red halo zone methods for each precipitation step to assess the effectiveness of the purification step (Harris, 2001; Islam and Roy, 2018)

3.11.3 Dialysis

Following the ammonium sulfate precipitation test, the enzyme with the highest activity was selected for dialysis. To prepare the dialysis bag, a 10 cm long bag was treated by washing it thoroughly with distilled water to eliminate glycerol, and to remove sulfide ions, it was placed in a solution of 0.2% $NaHCO_3$ and 0.1 mM EDTA. The bag was then boiled for 20 minutes, rinsed with distilled water multiple times, and further boiled in a 0.1 mM EDTA solution for 10 minutes to eliminate excess $NaHCO_3$. Once the pretreatment process was complete, one end of the dialysis bag was securely tied with thread, and the enzyme mixture was poured into the open end, which was then tied with thread as well.

An 80 mL phosphate buffer with a pH of 7 was added to a beaker, and the dialysis bag containing the enzyme was placed in the buffer with continuous stirring in a cool environment. The buffer was changed periodically until it no longer contained any SO_4- ions. The purified enzymes were transferred to clean screw-capped tubes and subjected to a Congo red assay to determine the presence of a halo zone, with the diameter of the zone measured for analysis (Gaur & Tiwari, 2015; Rosenberg et al., 2013)

3.11.4 Gel Filtration on Sephadex G-75

Gel-filtration chromatography was performed using Sephadex G -75 (Sigma Aldrich Pvt. Ltd) column material with a bed volume of 8-10 ml. A 2 g of Sephadex G-75 was weighed and suspended in 50 mL of 0.05 M sodium phosphate buffer (pH 7) was intermittently stirred for 2 hours and allowed to swell overnight. The swollen Sephadex G-75 slurry was then packed into a column measuring about 2.5 cm in diameter and 30 cm in length. To ensure efficient column packing and eliminate air bubbles, a slow flow of 50 mL phosphate buffer was used. The column was then equilibrated with sodium phosphate buffer (100mM, pH 7.0). Following equilibration, 1 mL of active fractions of dialyzed enzyme sample was carefully applied to the top of the column, followed by elution with a phosphate buffer. To maintain consistency, the flow rate was adjusted to 5-6 mL/hour. Throughout the process, 8 fractions comprising 2 mL, were systematically collected. Subsequently, both the enzyme activity and protein content were assessed for each fraction, following the procedure outlined in the previous section (Gaur & Tiwari, 2015).

3.12 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was employed to determine the molecular weight of the cellulase enzyme and to assess its purity (Laemmli, 1970). The active fraction, exhibiting the highest specific activity, obtained after gel filtration chromatography, along with crude enzyme, ammonium sulfate, and dialyzed product, was subjected to electrophoresis using SDS-PAGE on a 12% polyacrylamide gel. The experimental setup involved employing a vertical slab-gel unit Mini Protean II electrophoretic cell manufactured by Bio-Rad Laboratories. To start the experiment, a resolving gel was prepared in a 10 mL volume consisting of various components as shown in Table 6 was mixed gently and quickly introduced between two short plates with an in-built spacer mounted on the casting tray.

Table 6: Components of resolving gel for SDS-PAGE

Components of 12% resolving gel	For 5 mL	For 10 mL	For 15 mL
H_2O	1.6 mL	3.3 mL	4.9 mL
30% Acrylamide	2.0 mL	4.0 mL	6.0 mL
1.5 M Tris (pH 8.8)	1.25 mL	2.5 mL	3.8 ml
10% SDS	0.05 mL	0.1 mL	0.15 mL
10% $(NH4)_2S_2O_3$	0.05 mL	0.1 mL	0.15 mL
TEMED	0.002 mL	0.004 m	0.006 mL

The gel's surface was made smooth by layering isopropanol on top, which also prevented bubble formation. After the gel solidified, the excess isopropanol was carefully removed

by gently tilting the short plates upside down and using blotting paper to soak up the excess. Then, 3 mL stacking gel (5% polyacrylamide) prepared as shown in below Table 7 was layered onto the resolving gel.

Table 7: Components of stacking gel for SDS-PAGE

Components of 5% Stacking gel	For 1 mL	For 2 mL	For 3 mL
H_2O	0.68	1.4	2.1
30% Acrylamide	0.17	0.33	0.5
1.5 M Tris (pH 6.5)	0.13	0.25	0.38
10% SDS	0.13	0.02	0.03
10% $(NH4)_2S_2O_3$	0.01	0.02	0.03
TEMED	0.001	0.002	0.003

Before gel solidification, wells were created on the short plates by immediately inserting a Teflon comb and left undisturbed until solidification occurs (approximately 30 minutes). Once solidified, the comb was removed, and the wells were rinsed with buffer before the samples were loaded. Next, the slab containing the gel was inserted into the electrophoresis device, and tris-glycine electrophoresis buffer (running buffer) was added to both tanks. To prepare the protein samples, 20 µL of the sample was mixed with an equal volume of sample loading buffer in an Eppendorf tube. The mixture was then heated for 5 minutes to denature the proteins. The prepared samples, along with the protein marker, were loaded (10-15 µL per well) into each well according to the predetermined order. SDS- PAGE was conducted using a constant current supply of 90 V for 30 minutes to allow for protein stacking. After this initial stacking phase, the voltage was increased to approximately 120 V and the gel was run for approximately 3 hours. Following the completion of gel electrophoresis, the gel was carefully removed from the electrophoretic tank and detached from the gel plates. The gel was then immersed in Coomassie Brilliant Blue (a staining dye; Sigma company) and shaken gently at 100-120 rpm for approximately 10-12 hours. Subsequently, the gel was de-stained in a de-staining solution for 4 hours to overnight, effectively removing the excess dye and enhancing the visibility of the protein bands (Hafiz Muhammad Nasir et al., 2011; Sulyman et al., 2020)

3.13 Determination of kinetic parameters

The effect of substrate concentration on the reaction velocity of the purified cellulase was investigated using Carboxy Methyl Cellulose (CMC) as the substrate. Different concentrations of CMC (1, 2, 4, 6, 8, 10, 12 mg/mL) were used to incubate the purified cellulase. The enzymatic activity was measured under the standard conditions described above. The apparent kinetic parameters were determined by fitting data into Michaelis-Menten kinetic model using non-linear regression and the values were compared with that from a double reciprocal plot (Lineweaver-Burk plot). Therefore, the Michaelis constant (Km) and maximum velocity (Vmax) were determined. The obtained kinetic

parameters were used to characterize the cellulase enzyme and to optimize the reaction conditions for its application in various industrial processes (Gaur & Tiwari, 2015; Sulyman et al., 2020).

3.14 Application of Cellulase in bioethanol production

The optimal conditions obtained above were used to achieve the highest CMCase production by the isolate U1C4. Among the various lignocellulosic substrates tested, *Saccharum spontaneum* was selected as the most promising one for subsequent bioethanol production as cellulase production was found maximum in *Saccharum*, alongside rice straw, wheat straw, barley straw, and wood dust substrates. The glucose sugar resulting from the breakdown of lignocellulosic biomass (*Saccharum*) was then utilized for fermentation to produce ethanol using CDBT-2, an optimized strain of *Saccharomyces cerevisiae* of CDBT lab.

3.14.1 Inoculum preparation

To prepare the inoculum, a preserved yeast strain CDBT-2 (Gene Bank accession# MK910215y) was used. The inoculum was created by transferring a loopful of CDBT-2 agar culture into PYN (peptone, Yeast Extract, and Nutrient) media. It was then cultured in an orbital shaker incubator for 18 hours at a temperature of 30 ^{O}C and a pH of 5 (Joshi et al., 2019).

3.14.2 Pretreatment of biomass

Pretreatment of selected biomass*, Saccharum spontaneum* was carried out by hot water pretreatment method as described by Joshi et al (2019).

3.14.3 Saccharification using U1C4 isolate

The process of saccharification involves breaking down complex polysaccharides into simple fermentable sugars. In this case, a bacteria species was chosen because it can break down cellulose into sugars. Alternatively, saccharification can also be done using strong acids or commercially available enzymes like cellulase, pectinase, and xylanase, which can be purchased from pharmaceutical companies. However, using these enzymes can be expensive. To make the process more cost-effective, a locally isolated bacterial isolate U1C4 was used instead to convert the complex polysaccharide into simple sugars (Viera et al., 2019). In an experiment, two reagent bottles were used, one as a control and the other as a test. The test bottle contained optimized production media with pretreated *S. spontaneum* and freshly prepared U1C4 isolate culture, while the control bottle had the same conditions but without the U1C4 culture. Both bottles were incubated at 30°C for 7 days to allow the U1C4 isolate to saccharify the *S. spontaneum,* producing the reduced sugars needed for bioethanol fermentation. Samples were taken every 24 hours, and the production of reducing sugars was measured using a DNS assay. This meticulous monitoring allowed to identify the specific day when the maximum production of reducing sugars occurred. Subsequently, the highest amount of reducing sugars generated on this particular day was employed in the subsequent fermentation process.

3.14.4 Fermentation

In the production of bioethanol, a specially optimized strain of yeast named CDBT-2 derived from the CDBT laboratory at T.U. The fermentation process employed a stationary

method, where the yeast inoculum was added to a solution of glucose syrup obtained after the saccharification process. To maintain anaerobic conditions, the reagent bottles containing the glucose solutions were sealed tightly. These bottles were then placed in an incubator set at a temperature of 30 ^{O}C for 7 days. Throughout the fermentation process, samples were taken every 24 hours to estimate the presence the ethanol produced by using the NaOH-iodine precipitation method/ Iodoform test. The entire fermentation process lasted for 7 days. In addition to the NaOH-iodine method, each day's samples were also analyzed using an HPLC (High- performance Liquid Chromatography) machine to determine the concentration of ethanol more precisely. The purpose of this analysis was to identify the day during the 7-day period that produced the highest level of ethanol. To ensure accurate comparison, a control set was also prepared. This control set contained all the same components as the test samples, except for the yeast inoculum. By comparing the results of the test samples with the control, researchers could assess the impact of the yeast inoculum on ethanol production and determine its effectiveness in enhancing ethanol yield (Viera et al., 2019)

3.14.4.1 Iodoform test

The iodoform test was conducted to verify the presence of bioethanol produced by the bacterial isolate U1C4. In this test, 10 drops of the distillate were combined with 25 drops of iodine and 10 drops of NaOH in a test tube. After a short period, a cloudy formation, the appearance of a yellow precipitate, and the emergence of an antiseptic odor served as indications confirming the presence of bioethanol in the test tubes (Thomas et al., 2021).

3.14.4.2 HPLC analysis

High-Performance Liquid Chromatography (HPLC) was employed to analyze the actual concentration of bioethanol produced each day during a fermentation process spanning from day 1 to day 7. To accurately quantify the bioethanol compound, a series of calibration standards using absolute ethanol were prepared with varying concentrations of 1, 5, 10, 15, and 20 mg/mL. These calibration standards were filtered through a 0.2mm syringe filter and stored in individual HPLC vials. In addition, bioethanol samples were collected daily from the test fermentation bottle, as well as control samples from the control fermentation bottle. Each sample was placed in a separate Eppendorf tubes and centrifuged. The resulting supernatants were then filtered using a 0.2 mm syringe filter and stored in individual HPLC vials. For the concentration determination, an Aminex HPX-87H column (250*4) was utilized. The mobile phase consisted of 5 mM sulphuric acid, with a flow rate of 0.6/minute. The column temperature was maintained at 50°C. The concentration of bioethanol was determined based on the refractive index (RID) and a sample injection volume of 10 µL (Bio-rad, 2012).

3.14.4.3 Calculation of ethanol yield from reduced glucose during fermentation

In the process of monitoring the fermentation over a span of seven days, both the ethanol concentration and the diminishing glucose levels were assessed. To quantify the ethanol yield, it was calculated by dividing the ethanol concentration produced by the amount of glucose consumed. Ethanol yield (%) is thereby defined as the ratio of the ethanol concentration generated to the glucose consumed (Chang et al, 2018).The concentration

of reduced glucose was determined using the DNS method developed by Miller, employing glucose as a reference standard. Simultaneously, the ethanol concentration was ascertained through the High-Performance Liquid Chromatography (HPLC) method, as elaborated in section 3.14.4.2.

CHAPTER 4

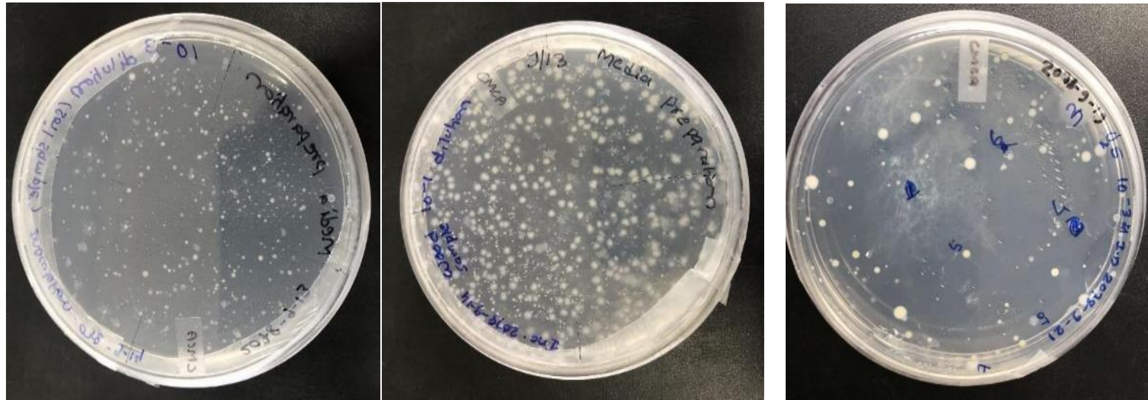
RESULTS AND DISCUSSION

The research involved the collection of different samples i.e. garden soil, cow dung, plant residue and rotten wood from different areas of Kathmandu i.e. Lainchour, Dhapasi, Chovar, and Kirtipur for the isolation of highly effective cellulolytic bacteria and explore their potential in the production of bioethanol from natural weeds and lignocellulosic biomass found useless around our surroundings. Among the samples collected, twelve distinct bacterial strains were identified as cellulolytic. The most potent strain was carefully selected during the experiment and subjected to optimization using different pretreated biomass substrates (*S. spontaneum*, rice straw, wheat straw, Barley straw, and wood) for enhanced cellulase production. The optimum condition was utilized in scaling up the enzyme production process to 3.5L in the Electrolab 360 fermenter. Following the successful scale-up process, the enzyme was employed in the fermentation and subsequent production of bioethanol. Among the various lignocellulosic biomass options, *Saccharum spontaneum* was determined to be the most suitable for this purpose, surpassing the other alternatives. Furthermore, the cellulase enzyme underwent partial purification using techniques such as ammonium sulfate precipitation, dialysis, and gel permeation chromatography. The purification process aimed to isolate and concentrate the enzyme for improved efficiency. Additionally, the SDS technique was performed to determine the molecular weight of the partially purified cellulase enzyme. Overall, the research focused on isolating potent cellulolytic bacteria, optimizing cellulase production by utilizing the enzyme for biomass hydrolysis, and ultimately producing bioethanol from *Saccharum spontaneum.* The cellulase enzyme was partially purified using various techniques, including SDS, to determine its molecular weight.

4.1 Isolation and Screening of cellulolytic organism

4.1.1 Isolation of cellulolytic bacteria

Distinct bacterial colonies with diverse morphologies were observed on the CMC media plates from different dilutions of soil samples (U1S, U2S, U3S), wood samples (U1W, U2W), and cow dung sample (U1C), as depicted in Figure 8. Eight distinct bacterial colonies were isolated from the soil samples, while 4 colonies were obtained from the rotten wood samples. Additionally, 4 colonies were isolated from the cow dung sample. Since the samples were initially inoculated on a selective cellulolytic agar medium containing CMC-cellulose as the carbon source, it was assumed that any bacterial colonies able to grow on the CMC medium were cellulolytic. However, it cannot be confirmed with certainty that all these bacterial colonies were cellulolytic, as there is a possibility of contamination during the handling process, resulting in the presence of non-cellulolytic organisms. These isolated colonies were purified and preserved in an agar slant and tested further for cellulolytic activities.



(A) (B) (C)

Figure 8: Mixed population of bacteria grown on Cellulolytic specific Carboxy Methyl Cellulose (CMC) Agar medium by spread plate technique (A) Soil sample (B) Wood sample (C) Cow-dung sample.

4.1.2 Screening for Cellulolytic Activity

For screening of cellulolytic activity, two methods were employed using plate hydrolysis method and well diffusion method.

Plate hydrolysis method

In this method, pure isolated bacteria samples obtained above were continuously streaked on the surface of the Congo-Red Agar media (CRAM) and incubated at 30°C for 48 hours. After incubation, a clear halo zone around the growing bacterial colony determines the cellulolytic activity of the isolates as shown in Figure 9. Among the 16 selected isolates, 12 isolates were able to hydrolyze cellulose present in the media which can be detected by the appearance of a clear white halo zone around their colonies.

(A) (B) (C)

Figure 9: Hydrolyzing zones produced by bacterial strains on cellulose Congo Red Agar media (A) Wood sample isolates (B) Soil sample isolates (C) Cow dung sample isolates.

In Figure 9, A represents four distinct colonies derived from wood samples streaked on Congo red agar media. B represents four different colonies from soil samples, and C

represents four distinct colonies from cow dung samples. All of these sample colonies exhibit the formation of a halo-zone on the Congo red agar media, indicating cellulolytic activity. However, the colonies derived from the cow dung samples demonstrate a higher level of cellulolytic activity compared to the other two samples. This is evident from the significant transformation of the dark red background around the cow dung colonies into a white halo zone, which covers almost the entire media. The breakdown of CM cellulose by the cellulase enzyme produced by these cow dung isolates is responsible for this change. Nevertheless, the colonies derived from the wood and soil samples also exhibit cellulolytic activity, albeit to a lesser extent.

Therefore, the use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Colonies showing a discoloration of Congo Red were taken as positive cellulose-degrading bacterial colonies and selected for the well diffusion method of screening.

Well Diffusion Method

The bacterial colonies showing halo zones on the primary screening method were further subjected to confirmation of cellulolytic activities. This was carried out by preparing crude enzyme extracts of the above selected isolated strains, poured in NA+1% CMC agar medium. After 48 hours of incubation, the plate was dispensed with 0.1% Congo red reagent and destained with 1M NaCl, and incubated for 15-20 minutes. The visualization of the clear zone with dark red background was observed. Out of 12 isolates, a clear zone of hydrolysis was observed in 10 strains with its halo zone diameter measurement listed in Table 8.

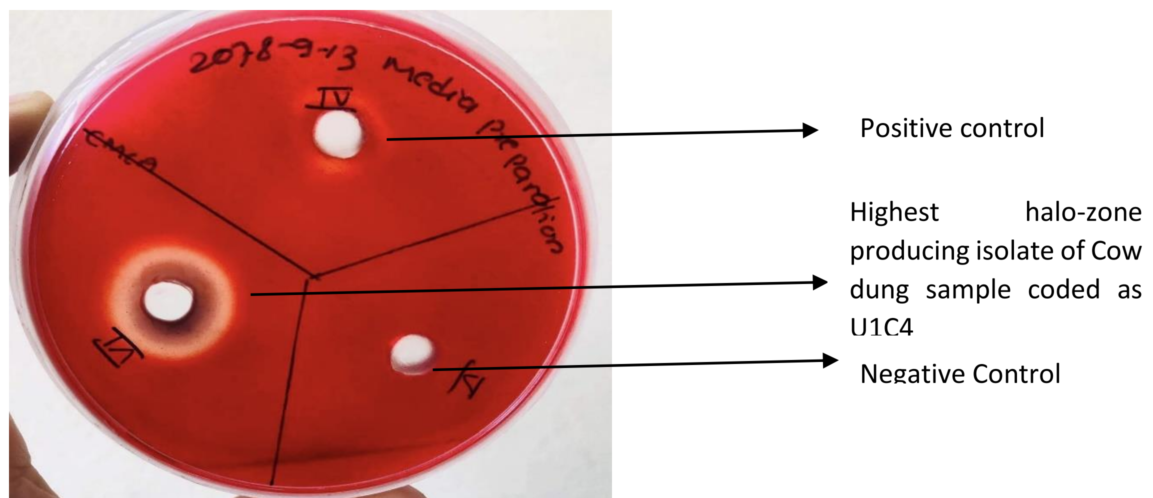


Figure 10: The degradation of cellulose medium, clear zone around the well with respect to crude enzyme source from isolate and CMC as substrate where symbol VI represents U1C4 sample, symbol V represents negative control, and symbol IV represents positive control.

Table 8: Well diffusion method for the analysis of cellulolytic capacity using Congo-Red reagent. The diameter of the halo zone produced by each organism around the well was noted.

Sample	Sample code(Isolate)	Colony number	Diameter of halo-zone (mm)	Dilution	Rank
Wood	**U2W2**	9	23	10-4	**2nd**
Wood	**U2W3**	2	12	10-4	**9th**
Soil first	**U1S1**	4	12.5	10-7	**8th**
Soil	U2S1	2	17	10-3	**5th**
Soil	U2S2	3	19	10-3	**4th**
Soil	**U2S3**	4	20	10-3	**3rd**
Cow dung	**U1C1**	1	15	10-4	**6th**
Cow dung	U1C2	2	11	10-4	**10th**
Cow dung	U1C3	3	13	10-4	**7th**
Cow dung	**U1C4**	4	25	10-4	**1st**

The results showed that the **U1C4** isolate from the cow dung sample achieved the highest halo-zone diameter of 25 mm, making it the top-ranked isolate. Following closely behind was the U2W2 isolate from the wood sample, which obtained a halo-zone diameter of 23 mm and was marked as the second-ranked isolate. In the third position was the U2S3 isolate from the soil sample, with a halo-zone diameter of 23mm. The fourth, fifth, and sixth positions were occupied by the isolates U2S2, U2S1, and U1C1, respectively, exhibiting halo-zone diameters of 19mm, 17mm, and 15 mm. The notable finding of the experiment was that the isolate from the cow dung sample displayed the highest halo-zone diameter. This result can be attributed to the presence of highly efficient cellulolytic organisms in the digestive systems of cows, which are known to possess the ability to break down cellulose. Cellulose is a complex carbohydrate found in plant materials such as straw, wood, cow dung, etc. The cow gut cellulolytic organisms present in the cow dung sample likely contributed to the higher halo-zone diameter observed in the U1C4 isolate. These organisms possess enzymes, such as cellulases, that aid in the breakdown of cellulose. As a result, they may have exhibited more efficient cellulose degradation, leading to a larger halo-zone diameter. The wood and soil samples also exhibited cellulolytic activity, although to a slightly lesser extent.

4.1.3 Analysis of reducing sugar produced

After conducting a qualitative test using a screening method, 6 superior cellulolytic isolates obtained above were selected for the quantitative test using the DNS assay. In the DNS test, the amount of reducing sugar produced by the isolates while breaking down CMC was measured at a specific wavelength of 540 nm as described by Miller, 1959. The activity of the enzyme was expressed in international units (IU/mL). The unit of cellulolytic activity was defined as the quantity of enzyme required to generate one micromole equivalent of glucose per minute. The standard curve for reducing sugar with glucose as a standard is given in Appendix II. Table 9 below presents the reducing values for the selected six isolates and it is evident from the table that the U1C4 isolate produced the highest amount of reducing sugar, reaching 618 µg/mL, compared to the other six isolates.

Therefore, isolate U1C4 was identified as the most efficient bacteria in terms of cellulase production and was selected for further identification procedures

Table 9: Reducing sugar production list of selected six isolates by DNS assay

Isolates codes	Reducing sugar produced (µg/mL)
U1C4	428.23
U2W2	419.04
U2S3	366.21
U2S2	347.08
U2S1	324 .32
U1C1	309.87

4.2 Identification of isolated strain

4.2.1 Colony morphology and microscopic observation of bacteria

Table 10: Colony morphology

S.N	Sample	Isolate code	Color	Shape	Size (mm)	Elevation	Opacity	Gram's staining
1.	Cow dung	U1C4	Off-white	Circular/ Slightly irregular	2-4 mm	Flat	Opaque	Gram-Positive

Based on the observations made during the colony observation of U1C4, the colony appeared either circular or slightly irregular in shape and had a diameter ranging from 2-4mm. The color of the colony was off-white. The colony had a flat elevation, meaning it did not protrude significantly from the surface it was grown on. The opacity of the colony was opaque. Also, the Gram staining result indicated that the bacteria present in the colony were rod-shaped and classified as Gram-Positive. Additionally, the spore staining result showed that the organism was capable of forming spores. Spores are specialized structures formed by certain bacteria as a survival mechanism under unfavorable conditions.

4.2.2 Molecular Characterization of the best strain

Bacterial isolate U1C4 was subjected to DNA extraction using a modified CTAB method (William et al., 2004). The extracted DNA exhibited a concentration of 176 ng/µL, with a purity ratio (absorbance 260/280) of 1.79. The 16S rDNA region of the isolate was amplified using Universal primers: 16sBakt341F as forward primer (CCTACGGGNGGCWGCAG) and 16sBakt805R (GACTACHVGGGTATCTAATCC) as reverse primer. Gel electrophoresis analysis of the resulting PCR product revealed a band of approximately 1550 base pairs, as depicted in Figure 11. A negative control, consisting of a reaction mixture without a template, was employed alongside a DNA ladder to facilitate

size comparison. Subsequently, the PCR product was sequenced and subjected to sequence homology analysis in Nucleotide BLAST.

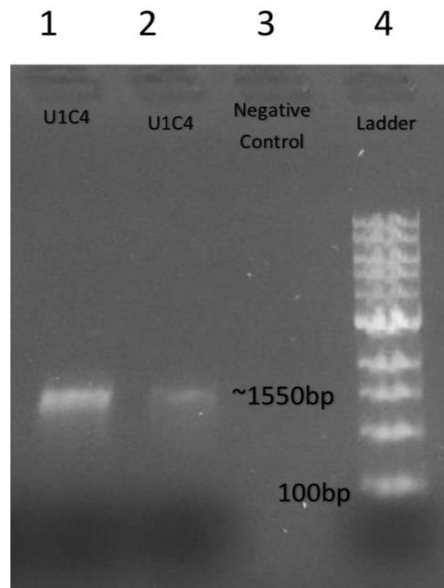


Figure 11: Gel electrophoresis of 16S rDNA specific PCR product of U1C4 bacterial isolate on 1% agarose gel. Lanes 1 and 2 represent PCR products of the U1C4 sample, Lane 3 is negative control and Lane 4 represents 1kb ladder.

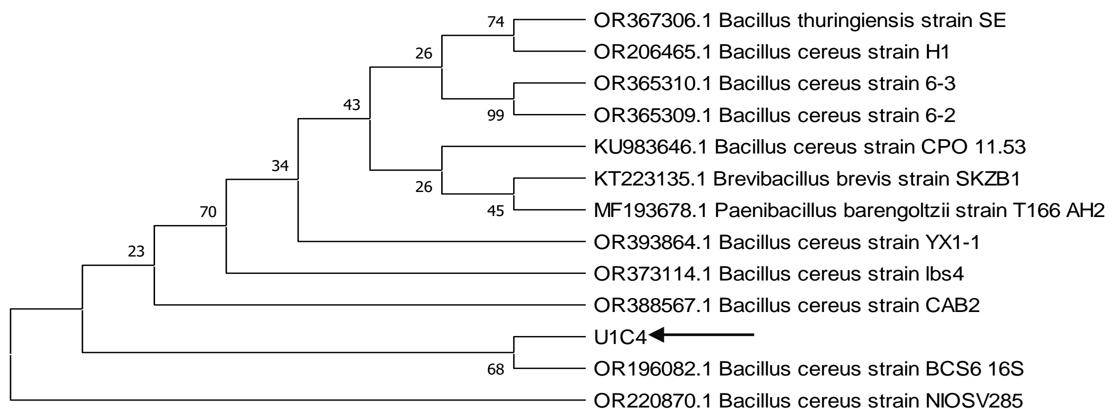
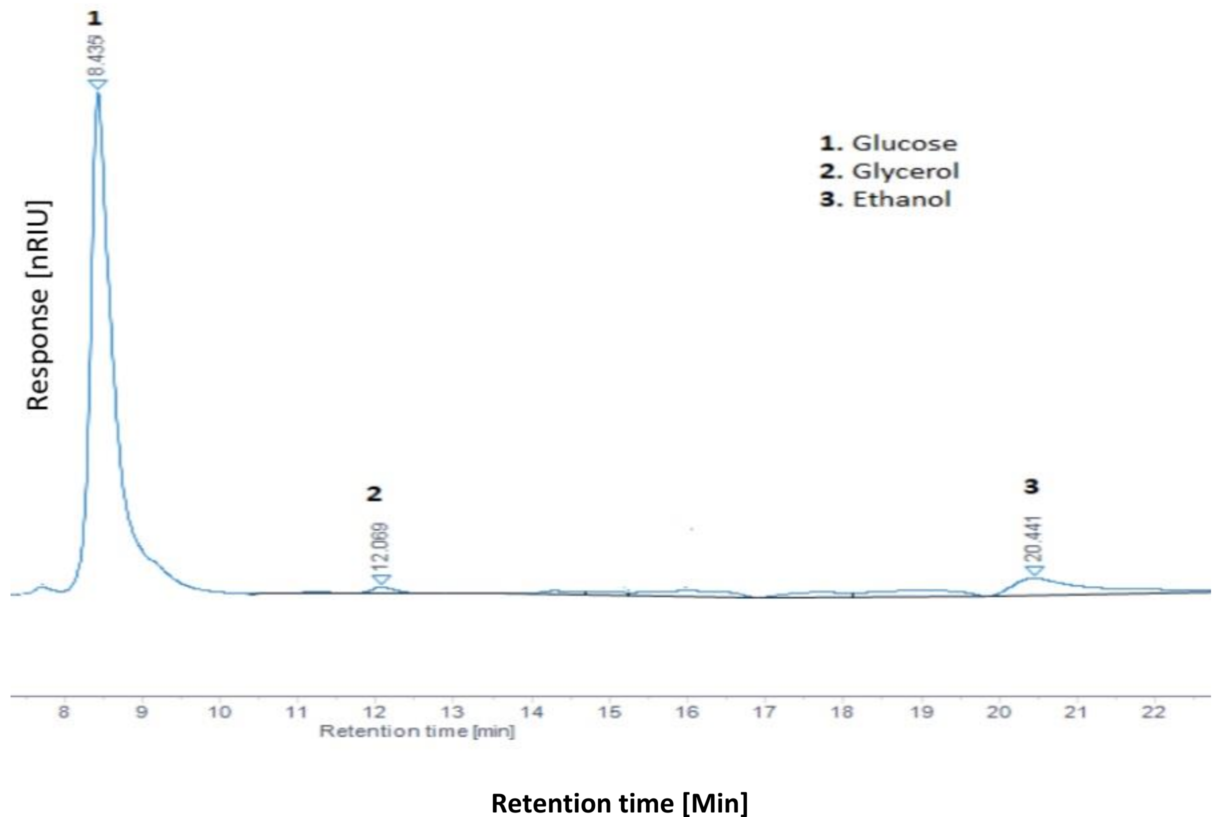


Figure 12: Phylogenetic tree constructed by Neighbour-joining (NJ) method based on 16S rDNA gene sequence comparison showing the position of U1C4 (*Bacillus Cereus*) and other related species of the genus using MEGA 11 software.

The isolate U1C4 showed 100% sequence similarity with *Bacillus* species, specifically *Bacillus cereus* strain CPO 11.53 from the NCBI Gene bank, according to phylogenetic tree analysis. Therefore, the isolate U1C4 was designated as *Bacillus cereus sp.* Strain U1C4. In the phylogenetic tree analysis, 12 species were compared, and the bootstrap value was determined based on 1000 replication. Bacillus cereus is a Gram-positive, rod-shaped, and facultative anaerobic microorganism that belongs to the phylum Firmicutes and the family Bacillaceae. It is commonly found in various environments such as cow-dung soil, water, and the gastrointestinal tracts of humans and animals. Schneider, 2017, reported that the optimal growth conditions for *Bacillus cereus* are temperatures between 28 and the pH

range of 4.9 to 9.3, which aligns perfectly with our research findings. This species is known to possess several enzymatic properties, including cellulases such as endoglucanase, exoglucanase, and β-glucosidase, which collectively break down cellulose into glucose units. These enzymatic properties make it useful for industrial purposes and as a probiotic in animal feed.

4.3 Analysis of sugar and other compounds in biomass substrates by High-Performance Liquid Chromatography (HPLC)



Retention time [Min]

Figure 13: Chromatogram of sugar and other compounds found in *Saccharum spontaneum* substrate using RI detector in HPLC

An analysis was conducted on various types of lignocellulosic biomass substrates, including *Saccharum spontaneum*, rice straw, wheat straw, barley straw, and wood. The analysis aimed to determine the presence and concentration of sugar and other organic compounds in the samples. High-Performance Liquid Chromatography (HPLC) with a specific column designed for carbohydrates and organic acids (Aminex HPX-87H) was used for the analysis. To quantify the different compounds in the biomass samples, calibration standards were analyzed using HPLC. The peaks in the resulting chromatogram were identified based on their retention time. Among the compounds detected in all five biomass samples were glucose, glycerol, and ethanol. Glucose was found to be present in the highest concentration in all the biomass samples, which is expected as it is the final simple degraded compound resulting from the breakdown of complex lignocellulosic biomass after pretreatment. Comparing the five substrates mentioned (*Saccharum spontaneum*, rice straw, wheat straw, wood dust, and barley straw) *Saccharum*

spontaneum had the highest amount of glucose (2.78mg/mL), followed by rice straw, wheat straw, wood dust, and barley straw, respectively. The concentration of glucose in the biomass samples was determined by comparing the peak areas of the samples with those of authentic standards, using an injection volume of 20 µL. Although glucose was quantified, the concentration of glycerol and ethanol was not calculated due to their minimal peak areas, making them nearly unnoticeable.

Table 11: Concentration of hydrolytic sugar found in biomass substrates in aqueous fractions obtained after digestion of biomass using acid treatment, characterized by HPLC-RID

Biomass sample	Compound detected	HPLC Concentration (mg/mL)
Saccharum spontaneum	Glucose	2.78
Rice straw	Glucose	1.77
Wheat straw	Glucose	1.11
Wood dust	Glucose	0.44
Barley straw	Glucose	0.29

An almost similar study was conducted by Silva et al., 2023 to analyze the sugar composition and concentration, specifically glucose, fructose, sucrose, trehalose, and xylose present in the sawdust of the flowering plant *Eucalyptus globulus.* Thermochemical liquefaction of sawdust is an attractive alternative for recycling this waste, as it leads to the production of bio-oil. The sugars obtained from the aqueous fraction of bio-oil can be highly valued and utilized by the industry for the production of sustainable materials (Silva et al., 2023).

4.4 Optimization of Cellulase production by U1C4 isolate

The most promising cellulase-producing isolate, *Bacillus cereus sp.* strain U1C4 strain was successfully identified. To enhance cellulase production, various factors such as pH, temperature, incubation time, biomass substrate (carbon source) and its concentration, nitrogen source, the concentration of peptone, and inoculum size were meticulously optimized in a step-by-step process. The optimization of growth media is crucial for advancing fermentation technology, as a cost-effective medium directly translates to reduced enzyme production expenses.

4.4.1 Effect of pH on Cellulase production

To optimize the pH conditions, a range of pH values from 4 to 10 was tested to investigate its effects on cellulase production. The isolated *Bacillus sp.* exhibited its maximum enzymatic production at a pH of 8.0 by all substrates (*S. spontaneum*, rice straw, wheat straw wood dust, and barley straw). *Saccharrum spontaneum* poses the highest CMCase

activity (0.425± 0.002 U/mL) followed by rice straw, wheat straw, wood dust, and barley straw respectively. While the isolate displayed minimum enzymatic activity at pH 4.0 and moderate enzyme activity at pH 6.0 and 7.0. The production level decreased significantly under alkaline conditions at pH 9, and very low enzyme production was observed at pH 10 in all substrates (Figure 14).

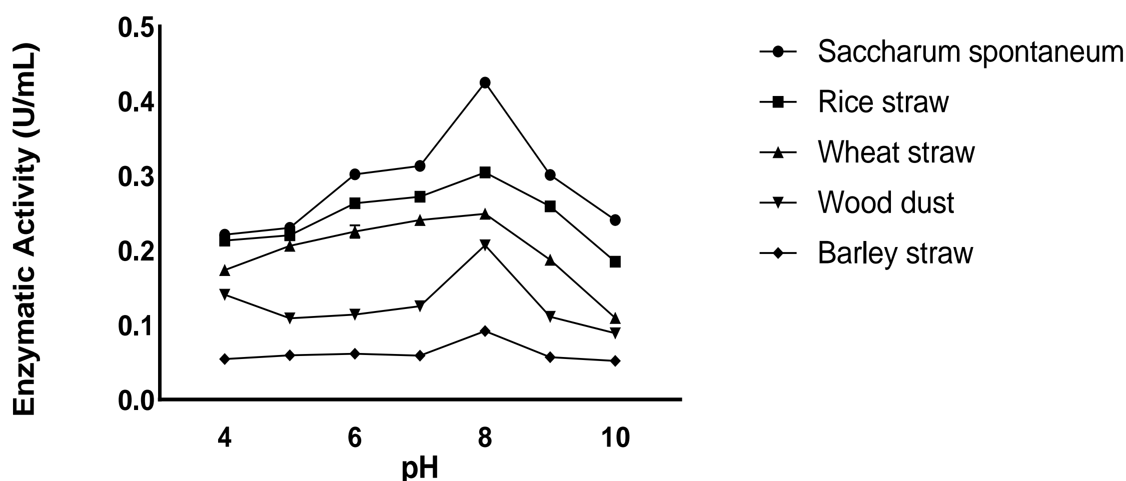


Figure 14: Effect of pH on Cellulase production by U1C4 isolate on different substrates.

4.4.2 Effect of temperature on Cellulase production

Temperature is a critical factor influencing the growth, physiology, and enzyme activity of microorganisms. In this study, various temperatures ranging from 20°C to 50°C were tested to examine their impact on the enzyme activity of *Bacillus sp.* The results revealed that the highest activity of the enzyme CMCase was recorded at 35 ^{O}C, reaching 0.468± 0.028 U/mL (Figure 15) by *S. spontaneum* biomass. Similarly rice straw also exhibited good enzyme activity of 0.422± 0.018 U/mL at 35°C. After that, it was observed a sharp decrease in enzymatic activity at 40°C in all substrates. Subsequently, enzyme activity continued to decline as the temperature increased beyond the optimum range, as temperatures above 45 ^{O}C led to thermal denaturation of the enzymes and consequently reduced enzyme activity. Furthermore, similar findings were reported in previous studies. For instance, *Bacillus pumilus* EWBCM1 exhibited a maximum cellulase production of 0.5851± 0.006 U/mL after 72 hours of incubation at 37^{0}C. Another study reported an exceptionally high cellulase production of 104.68 U/mL by *Bacillus sp*.BSS3 under pH 9 and 37^{0}C conditions using 1% CMC as a substrate.

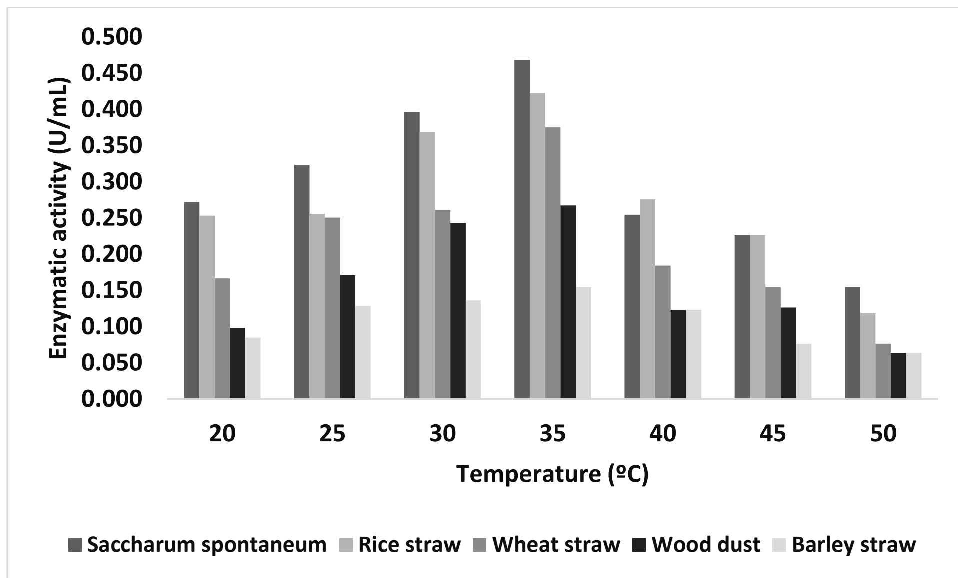


Figure 15: Effect of temperature on Cellulase production by U1C4 isolate on different substrates vs. temp.

4.4.3 Optimization of incubation time

The media optimized for cellulase production was tested at various time intervals (1, 2, 3 4, 5 and 6 days) after being inoculated with *Bacillus Cereus sp*. The highest cellulase activity (0.484± 0.04 IU/mL) was achieved after 3 days (72 hours) of incubation, as shown in Figure 16. However, beyond this point, the cellulase activity started to decline significantly. This decrease in cellulase production was attributed to either the depletion of essential nutrients or the accumulation of undesirable by-products in the fermentation media. These factors hindered further cellulase synthesis and led to a reduction in enzymatic activity. Another study by Das et al. also focused on cellulase production using thermophilic *Bacillus sp*. In their research, the maximum cellulase production of 2.817 µg/mg/min was attained after 3 hours of incubation.

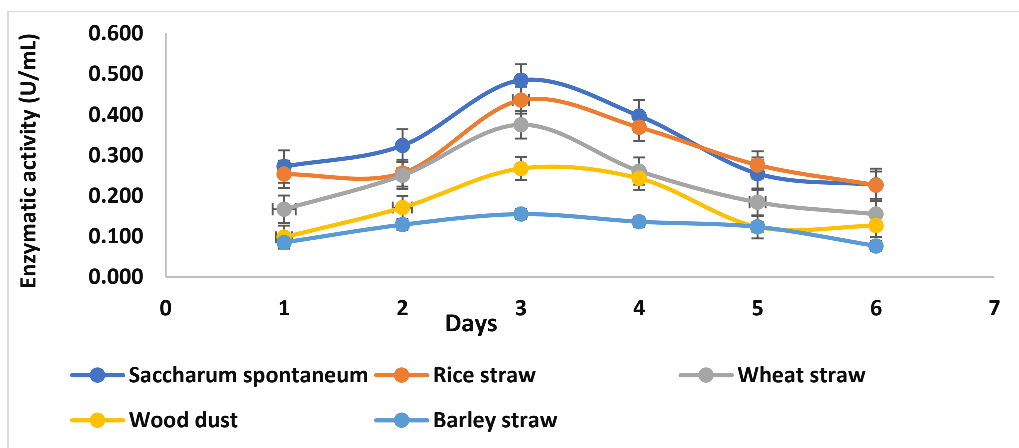


Figure 16: Effect of incubation time on Cellulase production by U1C4 isolate on biomass substrate.

4.4.4 Effect of carbon source

The most effective cellulolytic isolate, U1C4 was cultivated at 35 ^{0}C for 72 hours in a pH 8 basal media containing various nutrients and carbon sources supplemented with various biomass materials such as *Saccharum spontaneum*, rice straw, wheat straw, wood dust, barley straw, and CMC. The result showed that the highest CMCase activity, reaching 0.483± 0.041 IU/mL, was observed when the medium was supplemented with *Saccharum spontaneum* (as shown in Figure 17). This suggests that cellulase production is an inducible process, meaning it is stimulated by the presence of a specific substrate, which in this case acts as an inducer for the enzyme. Therefore, the type of carbon source used significantly influences the production of cellulase by *Bacillus sp*. U1C4 isolate.

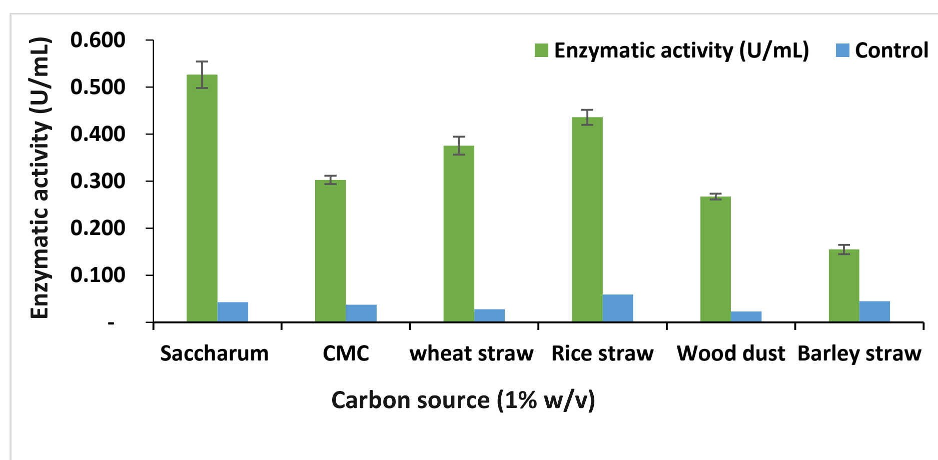


Figure 17: Effect of Carbon source on enzyme production by U1C4 isolate on different substrates

4.4.5 Effect of different concentrations of *Saccharum* spontaneum

The best carbon source with maximum cellulase production from *Bacillus sp.* U1C4 was found with biomass substrate, *S. spontaneum* at a concentration of 2% (w/v). Therefore to analyze the effect of different concentrations of *S. spontaneum* on cellulase production by the isolate, different concentrations of *S. spontaneum* (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, w/v) were used in the media. The maximum cellulase production with CMCase activity 0.488 ± 0.021 IU/mL was observed with a 2% (w/v) concentration of *S. spontaneum* (Figure 18).

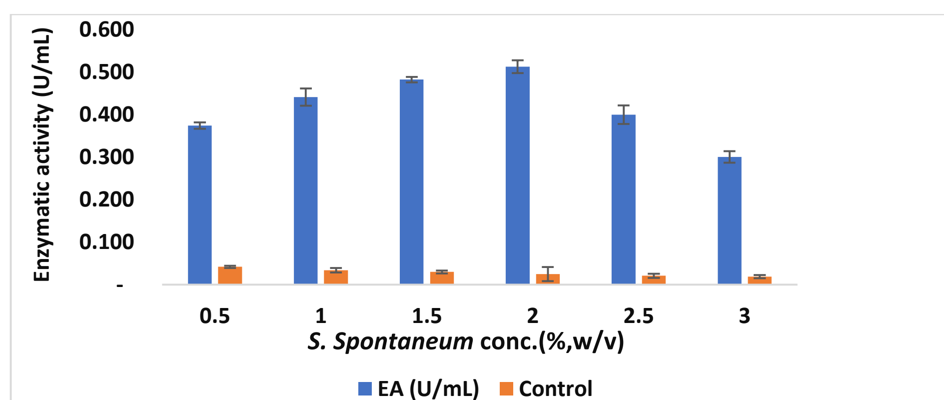


Figure 18: Effect of different concentrations of *S. spontaneum* on cellulase production.

These findings are consistent with previous studies conducted by another researcher. Abdullah et al., 2021) obtained the maximum CMCase activity of 1.225 U/mL at 2% conc. of *S. spontaneum* from *Taloromyces thermophiles* sp. when they used different concentrations of *S. spontaneum* ranging from 1% to 7% as the carbon source.

4.4.6 Effect of Nitrogen Source

The presence of nitrogen sources in the fermentation media is crucial for the production of cellulase, an extracellular enzyme. In the experiment, enzyme production was measured under various conditions using two different organic nitrogen sources, including peptone and yeast extract, as well as three inorganic nitrogen sources: ammonium sulfate, ammonium chloride, and urea, all at a concentration of 1% (w/v), on cellulase production was observed after 72 hours of incubation. Among these, peptone exhibited the highest enzymatic activity, reaching a level of 0.0470± 0.02 U/mL, as depicted in Figure 19. Interestingly, yeast extract also showed considerable enzymatic activity compared to the other nitrogen sources tested.

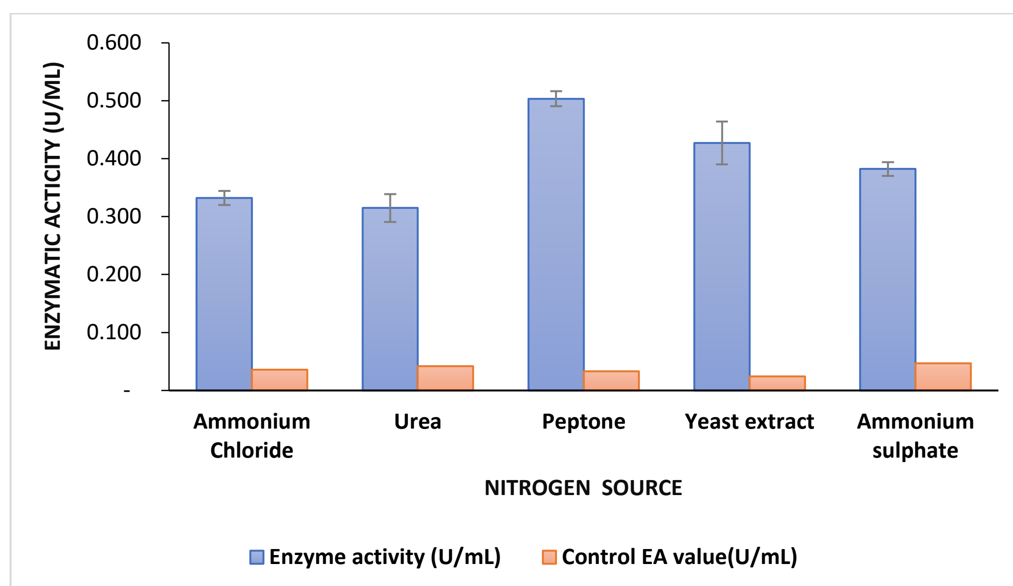


Figure 19: Effect of Nitrogen source on enzyme production, the isolate was incubated in basal media (Temperature 35°C, pH 8, *Saccharum* (2%) for 72 hours.

Similar observations were also made by some other workers in the past. The maximum enzyme activity of 2.910 µg/mg/min was achieved from *Bacillus sp.* when peptone was utilized as a nitrogen source by Das et al. Recently, Lugani et al. showed similar results with maximum CMCase: 4.72 IU/mL production from newly isolated *Bacillus sp.* Y3 in basal medium supplemented with peptone as a nitrogen source. These findings highlight the significance of selecting appropriate nitrogen sources in the fermentation media to optimize cellulase production.

4.4.7 Effect of different concentrations of peptone

Peptone has been identified as the most effective nitrogen source for achieving optimal cellulase production from the *Bacillus sp.* U1C4 isolate. To determine the effect of different concentrations of peptone ranging from (0.5%-3%, w/v), cellulase production

was studied. The results revealed that the highest cellulase production, reaching a CMCase activity of 0.486± 0.005 U/mL, was obtained with a peptone concentration of 1% (w/v), as depicted in Figure 20.

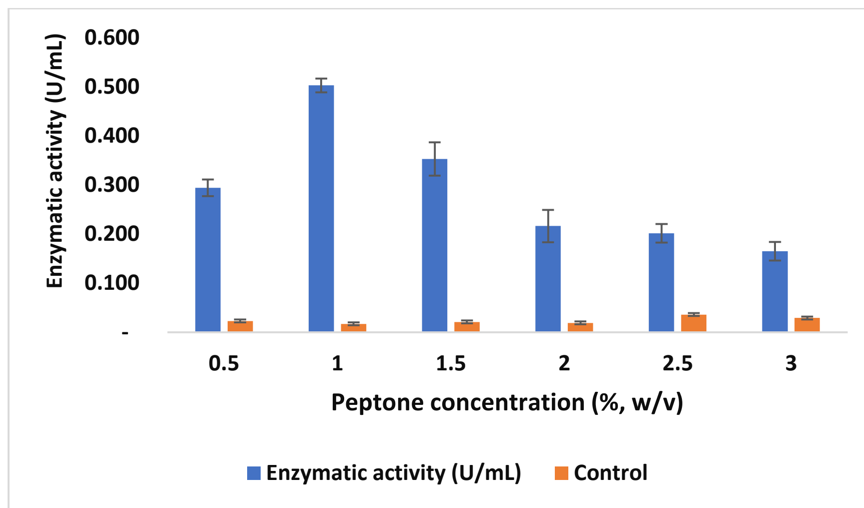


Figure 20: Effect of different concentrations of peptone on cellulase production

Similarly, the research conducted by Das et al (2010) has also shown that peptone serves as a favorable nitrogen source for enhancing cellulase production in Thermophilic *Bacillus sp.* isolated from Cow dung. In a separate study conducted by Paudel and Qin (22), they reported that *Bacillus sp*.K1 achieved maximum cellulase production at a peptone concentration of 1% (w/v).

4.4.8 Effect of inoculum size

The effect of different concentrations of *Bacillus cereus sp*. strain U1C4 culture (0.5%, 1%, 2%, 3%, 4% and 5% v/v) as inoculum on cellulase production was investigated. The results revealed that the optimal inoculum concentration for cellulase production was determined to be 2% (v/v), which resulted in the highest CMCase activity of 0.465± 0.006 U/mL) (Figure 21).

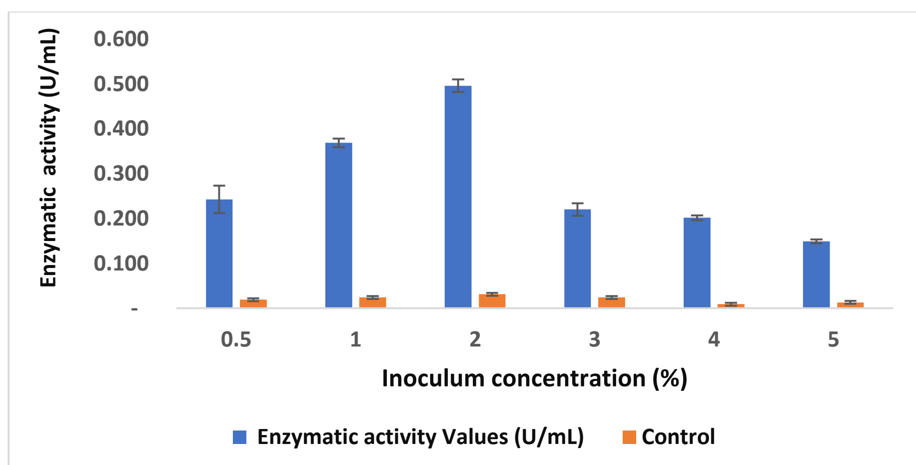


Figure 21: Effect of inoculum concentration on cellulase production

However, beyond this optimum concentration, the enzyme activity sharply declined due to reduced microbial growth caused by increased competition for space and nutrients among cells. These factors also affected the length of the stationary phase, leading to a loss of enzyme activity due to the accumulation of toxic products and secondary metabolites. These findings are consistent with previous studies conducted by Acharya and Chaudhary (25), who reported a maximum cellulase production at an inoculum size of 2% (v/v) using CMC from *Bacillus llichenformis* WBS1. Similarly, Shankar and Isaiarasu observed that a 2% (v/v) inoculum size was optimal for maximum cellulase production by *Bacillus pumilus.* (Shankar & Isaiarasu, 2011)

4.5 Scale-up of enzyme production using optimized conditions.

Scaling up the production of enzymes is essential for achieving economical and industrial-level production of enzymes under optimized conditions. The enzyme production using *Bacillus cereus sp*. strain U1C4 was scaled up to 3.5 L production media in Electrolab 360 fermenter located at the Central Department of Biotechnology in Kirtipur, Nepal, and was done in optimized conditions. The growth conditions were optimized, including a temperature of 35°C, pH of 8, and dissolved oxygen (DO) maintained at 100. The fermentation process lasted for 7 days, with agitation (100 rpm) and without agitation for separate durations while keeping other parameters constant. The results of enzyme production are as follows:

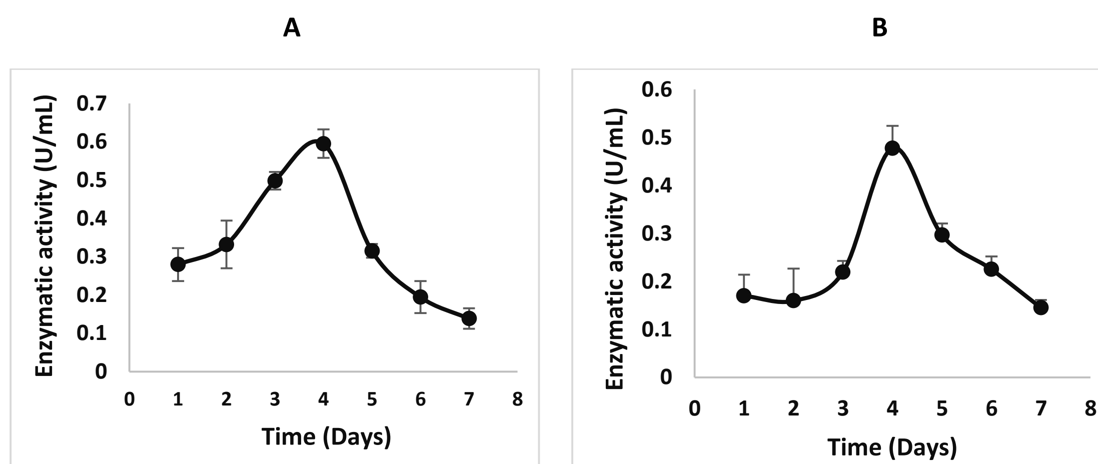


Figure 22: Enzymatic activity of cellulase enzyme produced in Electrolab 360 fermenter. (pH 8, temperature 35°C, Substrate: *Saccharum* (2%), Nitrogen source: Peptone(1%) , inoculum size (2%) (A) Represents enzymatic activity with agitation (B) represents enzymatic activity without agitation.

Two graphs depict the enzyme activity of *Bacillus cereus sp*. strain U1C4 with agitation and without agitation while maintaining other parameters constant in both setups. The analysis showed that the highest activity of the enzyme CMCase was observed on the 4th day of incubation in both fermenters. Comparing the two conditions, the fermenters with agitation exhibited a greater CMcase activity (0.595± 0.021 U/mL) compared to the fermenter without agitation (0.4775± 0.013 U/mL). The reason behind this difference

could be attributed to the improved distribution of dissolved oxygen and more efficient nutrient uptake, leading to higher enzyme production. Additionally, agitation in the fermenter promotes better mixing, prevents substrate gradients, eliminates inhibitory byproducts, and reduces shear stress, resulting in increased enzyme activity compared to a non-agitated fermenter (Das et al., 2010). The results showed moderate enzyme activity from the first to the third day of incubation, with maximum production achieved on the 4th day. However, as the days of incubation increased, the enzyme production gradually declined, reaching very low levels on the 7th day in both setups. Previous studies have suggested that as the cellulose substrate is consumed over time, the availability of substrates for the enzymes to act upon decreases, leading to a natural decline in cellulase activity. Furthermore, factors such as product inhibition, enzyme denaturation, microbial competition, and genetic regulations may contribute to the decrease in enzyme production over time as well.

4.5.1 Comparative analysis of enzyme production in the fermenter and Erlenmeyer flask

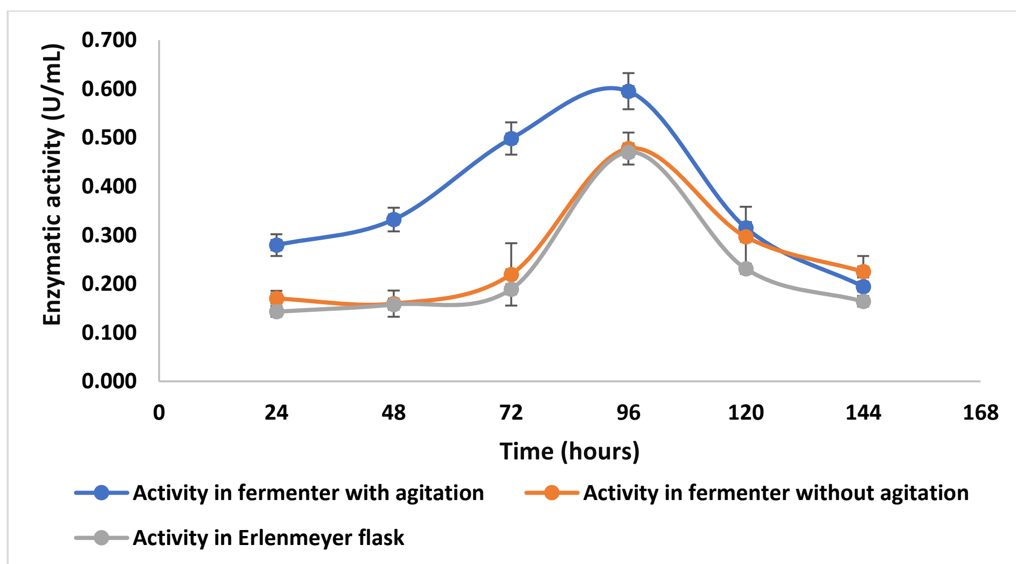


Figure 23: Comparison of enzyme activity in the fermenter with agitation and without agitation and 250 mL Erlenmeyer flask.

In our study on enzyme production, we compared three different setups: a 250 mL flask with 50 mL of media, and a fermenter with 3.5 L of media with agitation and without agitation. The results, shown in Figure 23, revealed that enzyme production was higher in the fermenter with agitation (0.595± 0.021 U/mL) compared to the production in a 250 mL conical flask (0.484± 0.04 U/mL). However, when comparing enzyme production in a non-agitated fermenter and a conical flask, the results were similar, with optimal enzyme production of 0.478± 0.013 U/mL and 0.470±0.02 U/mL respectively. Both setups reached their optimum production after four days. Our findings suggest that agitation plays a crucial role in enzyme production by facilitating better mixing and creating a homogenous environment. This helps to prevent the accumulation of toxic by-products or gradients

that could negatively affect enzyme activity. It is important to note that we did not optimize the agitation rate in our study due to the absence of a shaker incubator in our laboratory. Therefore, the incubation was done without agitation during the optimization for maximum enzyme production by U1C4 isolate.

4.6 Production, purification, and quantification of cellulase

Once the optimal conditions for producing maximum cellulase enzyme were determined, the isolated *Bacillus cereus* sample was cultured in a fermentation medium containing 2% pretreated *S. spontaneum* powder as growth supported substrate. The culture broth thus produced was used to prepare crude enzymes using the method described earlier. The crude enzyme, precipitated, dialyzed, and gel filtrated of all types was then quantified using the Bradford assay and CMCase assay to determine its protein content and enzyme activity. The crude cellulase exhibited a total activity of 39.12 U/mL and a specific activity of 0.7409 U/mg (Table 12). As the crude enzyme underwent each purification step, the total activity decreased which was accompanied by a corresponding increase in the specific activity. The ammonium sulfate precipitation test showed that 60% saturation of salt precipitation resulted in the highest activity (Figure 24), with a specific activity of 0.813 U/mg and a 1.09-fold purification.

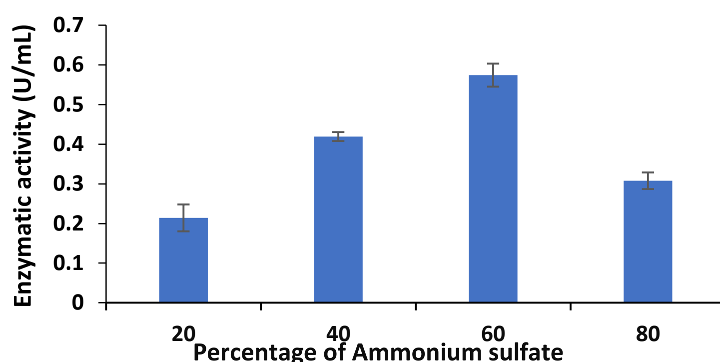


Figure 24: Ammonium sulfate for salt precipitation of crude cellulase

Table 12: Activity of cellulase enzyme at different purification steps.

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude cell-free extract	80	52.8	39.12	0.7409	100	1
60% ammonium sulfate ppt.	10	7.06	5.74	0.813	14.67	1.09
Dialysis	6	2.52	3.516	1.395	8.98	1.88
Sephadex G-75	3	0.45	2.112	4.693	5.4	6.34

After dialysis with buffer changes, the partially purified cellulase exhibited a specific activity of 1.395 U/mg and 1.88- fold purification. The purified cellulase obtained after gel filtration on Sephadex G-75 exhibited a 6.34-fold purification and a specific activity of 4.693 U/mg (Table 12). Therefore fourth successive purification achieved a purification factor of 6.34-fold resulting in a final recovery of 5.4% of the enzyme with a specific activity of 4.693 U/mg. Throughout the purification process, the specific activity of the enzyme increased, indicating improved efficiency, while the overall protein yield decreased. This decrease in protein yield can be attributed to the loss of enzymes during the purification steps.

4.6.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile of purified cellulase

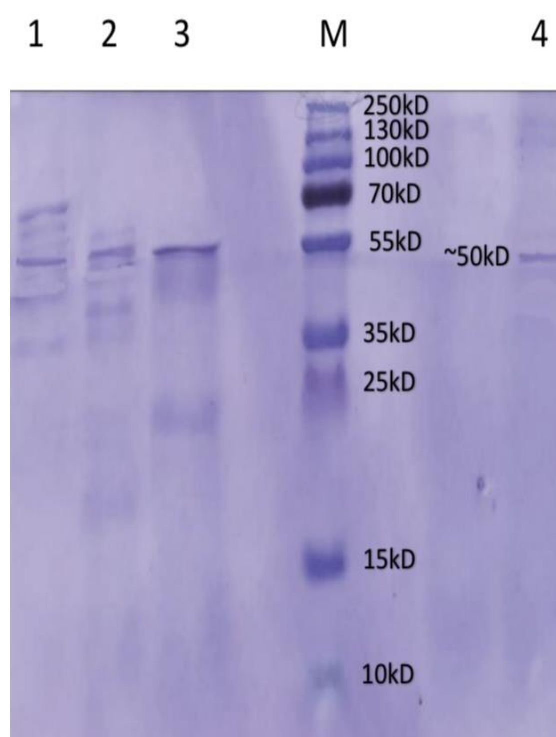


Figure 25: A SDS-PAGE of cellulase enzyme extracted from isolated *Bacillus cereus* sp. strain U1C4. (1= Crude enzyme , 2= Precipitated enzyme 3= Dialyzed enzyme, 4= Column purified enzyme(Approx 50 kDa), and M= Protein marker (Promega, USA))

The SDS_PAGE analysis of cellulase produced by *B.cereus* sp. strain U1C4 is presented in Figure 25. The molecular weight of the purified enzyme (Lane 4) was determined using 12% SDS PAGE in which a distinct band representing the enzyme was observed at approximately 50kDa when compared to the Broad Range Protein Molecular Weight Marker (Promega, USA). Lane 1 displayed the crude enzyme, Lane B contained the ammonium sulfate fraction, Lane 3 represented the dialyzed enzyme and Lane 4 contained the fraction obtained after gel filtration chromatography. Previous studies have reported that cellulases generally exhibit molecular masses ranging from 31 to 94 kDa (Endo et al., 2001). Similarly, the CMcellulase protein in *B. Pumilus* EB3 was reported to have a molecular weight range of 30 to 65 kDa (Ariffin et al., 2008). Moreover, the

cellulases from *B. lichenformis* were found to have protein sizes larger than 37 to 43 kDa (Bischoff et al., 2006). Other examples include the endo-1,4- β glucanases from the moderate thermophilic strain *B. lichenformis* ATCC 14580, which had a molecular weight of 52.2 kDa (Aftab et al., 2012) and the purified cellulase from *Bacillus amyloliquefaciens* isolated from rice hulls (Lee et al., 2008) had a molecular weight of 54kDa. Likewise, putative cellulases from *Bacillus subtilis*, such as CelL15 and CelL73, showed protein band sizes of 54 and 27 kDa, respectively (Li et al., 2009). Therefore, based on these similarities, comparisons, and examples mentioned above, it can be concluded that the enzyme under study exhibits characteristics similar to endoglucanases or putative cellulases, rather than possessing a complete system consisting of exoglucanases, endoglucanases, and beta-glucosidases.

4.7 Determination of kinetic parameters

The purified cellulase obtained from the selected cellulolytic *Bacillus sp*. was used to study its enzymatic behavior by determining the Michaelis-Menten kinetic constants, K_m and V_{max}. This was achieved by testing the enzyme's activity at various concentrations of CMC substrate. The data obtained from these experiments were then analyzed using the Michaelis-Menten kinetics model, and a Lineweaver-Burke plot was utilized for a more accurate determination of K_m and $V_{max.}$ The Lineweaver-Burk plot allowed us to calculate the values of K_m and V_{max}, which were found to be 5.16 mg/mL and 33.22µmol/ml/min, respectively (Figure 27). These values fell within the expected range of Michaelis-Menten kinetics further confirming the validity of our approach. In comparison, the predicted K_m and V_{max} values obtained from Figure 26 were approximately 3.91 mg/mL and 26 µmol/mL/min, respectively.

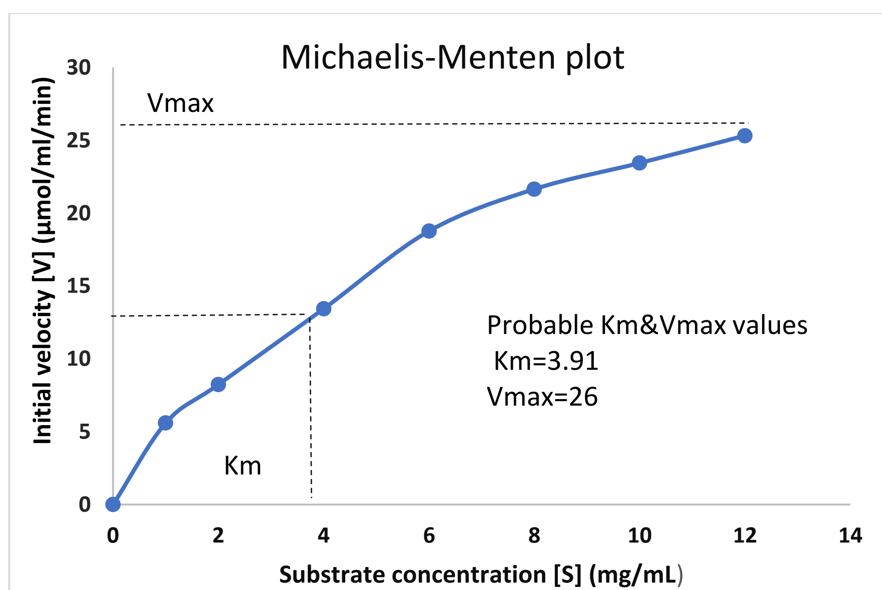


Figure 26: Determination of K_m and V_{max} for purified cellulase through Michaelis-Menten kinetics.

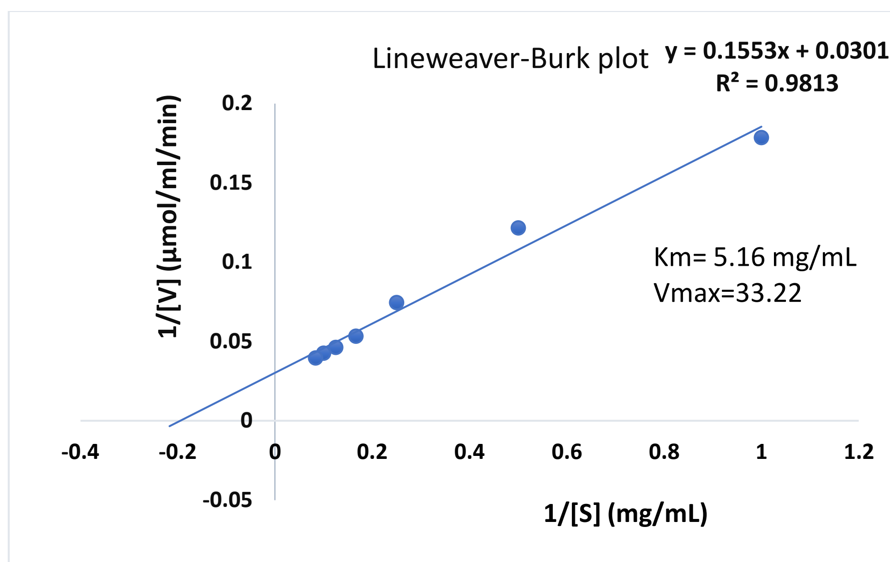


Figure 27: Lineweaver-Burke plot of initial velocity data of *Bacillus cereus sp*. strain U1C4 on different concentraion of CMC.

The results of our kinetic analysis revealed that the enzyme displayed a strong affinity for the substrate, indicated by a low K_m value of 5.16 mg/mL. This means that the enzyme could achieve half of its maximum rate (V_{max}) at a relatively small concentration of the substrate. These findings were consistent with a previous study by Afzal et al. (2010), where similar Km (4.1 mg/mL) and V_{max} (25 µmol/mL/min) values were reported for *Bacillus sp.* using CMC as the substrate. Additionally, the K_m value, 8.73 mg/mL, resembled the CMCase activity from *Paenibacillus polymyxa* (Kumar et al., 2012) but with a lower V_{max} of 17.805 mM/ml/min. This variation in V_{max} might be attributed to differences in the activity of the purified enzyme's endoglucanase under different optimal conditions or due to genetic variability among different species. Therefore, the strong substrate affinity of the enzyme, as demonstrated by the low K_m value, highlights its potential for efficient catalysis at relatively low substrate concentrations. These findings contribute to our understanding of cellulase kinetics and can be valuable in various biotechnological applications involving cellulose degradation.

4.8 Application of Cellulase in bioethanol production

Bioethanol was produced from *Saccharum spontaneum* biomass through saccharification and fermentation processes. The saccharification was carried out using *Bacillus cereus sp*. strain U1C4, while fermentation was performed using *Saccharomyces cerevisiae* (CDBT2) yeast strain optimized by Joshi et al. (2019). During saccharification, periodic samples were collected to measure residual sugar levels. The results (Table 13) indicated that the highest concentration of reducing sugar (767±0.006 µg/mL) was achieved on the 3rd day of the process. Initially, the hydrolysate contained 410.24 ± 0.043 µg/mL of glucose, which gradually increased until the 3rd day, after which there was a minor decline in released sugar concentration.

Table 13: Glucose concentration (µg) during saccharification

Days of incubation	Concentration of reduced glucose (µg/mL)
1	410.24 ± 0.043
2	625.35 ± 0.013
3	767± 0.006
4	691.89 ± 0.02
5	613.77 ± 0.008
6	517.89 ± 0.006
7	510.09 ± 0.071

The reducing sugar generated on the 3rd day was subsequently utilized for fermentation using the yeast strain CDBT-2. This fermentation process was carried out under anaerobic conditions for 7 days. Samples were collected at 24-hour intervals, and ethanol content was determined using the NaOH-iodine precipitation method. The actual ethanol concentration was calculated using an HPLC machine, with absolute ethanol serving as the standard.

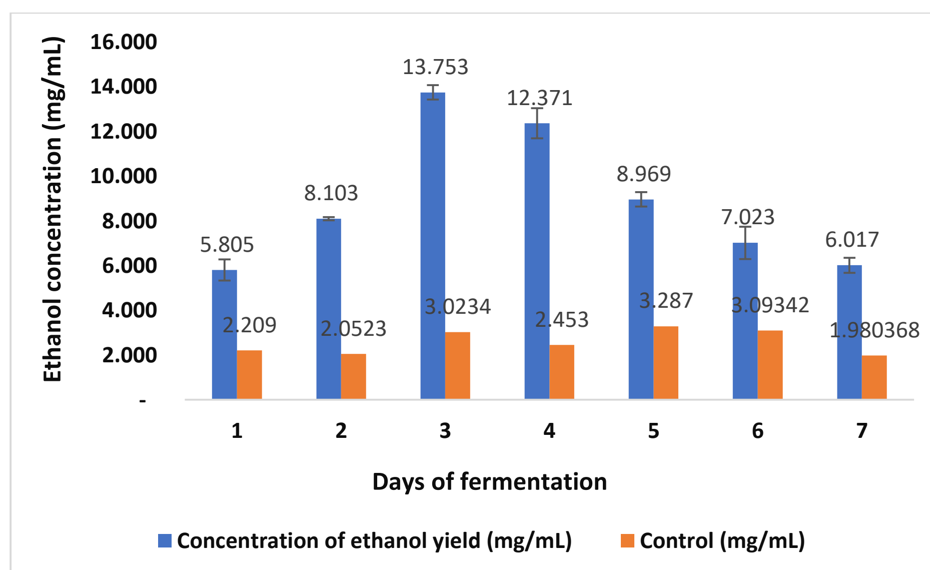


Figure 28: Estimation of ethanol production vs. time interval. Ethanol content was determined using the NaOH-iodine precipitation method. The actual ethanol concentration was calculated using an HPLC machine, with absolute ethanol serving as the standard.

As for ethanol production (Figure 28), it showed an increasing trend from the 1st day to the 3rd day, followed by a small decrease on the 4th day (9.92± 0.093 mg/mL). However, after the 4th day, there was no significant increase in ethanol production. The decrease in

ethanol concentration could be attributed to ethanol evaporation or degradation into other byproducts. Adeboye et al. (2015) previously reported that ethanol assimilation occurs after the diauxic shift, which is the point when all the glucose has been consumed. The highest ethanol yield observed in our study was 10.73± 0.045 mg/mL on the 3rd day (obtained by subtracting the control value of 3.0234 mg/mL from the test value of 13.753 mg/mL), consistent with the release of reducing sugars during saccharification. It is important to note that variations in the obtained results compared to other research studies may be attributed to factors such as different growing locations, seasons, stages of harvest, harvesting methods, and analytical procedures used.

4.8.1 Calculation of ethanol yield from reduced glucose during fermentation

Table 14: Percentage of ethanol yield in terms of reducing of sugar

Fermentation days	Reduced glucose (mg/mL	Initial reduced glucose (mg/mL)	Glucose consumed	Ethanol Conc. (mg/mL)	Ethanol yield (%)
1	17.709	22.06	4.351	3.6	82.65
2	14.546	22.06	7.514	6.05	80.53
3	7.046	22.06	15.01	10.73	71.46
4	6.562	22.06	15.5	9.92	64
5	6.453	22.06	15.61	5.68	36.41
6	5.064	22.06	17	3.93	23.12
7	4.59	22.06	17.47	3.04	17.38

S. cerevisiae is an economically efficient and viable choice for ethanol production due to its rapid reproduction, making it suitable for large-scale processes. In batch cultures with limited nutrients, this yeast quickly converts sugars into ethanol. Based on the results obtained during fermentation, *S. cerevisiae* (CDBT-2) initially benefits from cellulase activity, which increases the total reducing sugar content to 17.709 mg/mL. As fermentation progresses, yeast consumes these sugars to produce ethanol, causing a sharp decrease to 4.59 mg/mL by the seventh day and thus lead to the highest ethanol yield, 82.65%, occurs after 24 hours, when ample reducing sugar is available. Ethanol yield then gradually decreases with longer fermentation time, dropping sharply to 17.38%. This decline is attributed to diminishing reduced glucose levels, as some of the glucose may be consumed by the cellulolytic isolate U1C4 itself for their growth and survival because of increased cell biomass over time. This observation aligns with Chang et al.'s (2018) study, which found that high initial glucose concentrations initially boost ethanol yield to 8.3 g/L during first 18 hours of fermentation and then gradually decreased over time.

CHAPTER 5

SUMMARY

The demand for sustainable and renewable energy sources has increased, leading to extensive research in finding eco-friendly alternatives to traditional fossil fuels. Bioethanol derived from lignocellulosic biomass has shown great promise as a viable biofuel option due to its renewable nature and reduced greenhouse gas emissions. However, the process of efficiently converting lignocellulosic biomass into fermentable sugars, a crucial step in bioethanol production, remains a challenging and expensive bottleneck.

This thesis aimed to address this challenge by investigating cellulase-producing bacteria from various environmental sources. The study focused on isolating, screening, and optimizing the most efficient cellulolytic bacteria that can break down lignocellulosic agricultural wastes (such as rice straw, wheat straw, barley straw, and wood) and wild weeds (*Saccharum spontaneum*), which are abundantly found in the waste lands of Nepal. The goal was to select and utilize the most potent cellulolytic strain for the efficient production of bioethanol from cellulosic substrates.

The study involved isolating bacterial strains from different sources like garden soil, compost, plant residues, cow dung, and rotten wood to explore the diversity of cellulase-producing microorganisms. In the investigation, a total of 16 morphologically different cellulose-degrading bacterial isolates were successfully cultured on cellulolytic selective CMC medium. The bacteria were then screened primarily by plate hydrolysis and further confirmed by well diffusion method along with Congo red staining and NaCl treatment. Among 12 pure culture of bacterial isolates, 10 exhibited robust cellulolytic activity. Notably, the cow dung-derived U1C4 isolate displayed the highest cellulolytic potential and was chosen for further characterization. This isolate, identified as *Bacillus cereus spp.* was confirmed through morphological, biochemical, and phylogenetic analyses and designated as *Bacillus cereus spp.* strain U1C4. Subsequently, the potential of this bacterial strain for bioethanol production was investigated using five different lignocellulosic wastes. To break down the complex recalcitrant structure of lignocellulose, a hot-water pretreatment method was employed, effectively releasing cellulose and hemicellulose from lignin. U1C4 isolate was subjected to optimization studies using the mentioned biomass substrates separately to enhance its cellulase production capabilities. Various culture conditions and nutritional factors were evaluated to maximize enzyme yields. The optimum temperature and pH conditions for maximum CMCase activity were 35°C and pH 8, respectively. Also, best Carbon and Nitrogen source was found as *Saccharum spontaneum* and peptone in their optimum concentrations of 2% and 1% respectively. The inoculum size (2%) and time of incubation (72 hours) were also optimized. Among the five biomass substrates, *Saccharum spontaneum* showed the highest cellulase production, followed by rice straw, wheat straw, wood dust, and barley straw.

The crude cellulase enzyme produced by U1C4 isolate using *Saccharum spontaneum* as the carbon source under all optimized conditions was partially purified through ammonium sulfate precipitation, dialysis, and gel permeation chromatography using Sephadex G-75. At the last stage of purification by Sephadex G-75, cellulase was purified to 6.34-fold with a yield and specific activity of 5.4% and 4.693 U/mg respectively. The enzyme's profiles were characterized through zymography and its molecular weight was found to be approximately 50 kDa on 12% SDS-PAGE analysis. Enzyme kinetics studies were conducted, revealing the K_m and V_{max} values as 5.16 mg/mL and 33.22 µmoL/mL/min, respectively. Upon scaling up, the optimized *Bacillus sp.* demonstrated enhanced enzyme production in an Electrolab fermenter 360 compared to a 250 mL conical flask. By the fourth day of incubation, CMCase activity reached to maximum (0.595 ± 0.021 U/mL) in the fermenter, surpassing the 0.484 ± 0.04 U/mL cellulase activity observed in the conical flask. This indicates that the optimization process led to a significant increase in enzyme production.

In the final phase of the research, bioethanol production using *Saccharum spontaneum* was carried out. The aim was to release fermentable sugars from cellulose and hemicellulose components using the cellulase enzyme produced by the selected isolate U1C4. Ethanolic fermentation was done using well- optimized CDBT-2 strain of *Saccharomyces cerevisiae* (which had been previously optimized by my seniors) after the saccharification of *S. spontaneum* by U1C4 strain. The anaerobic fermentation was carried out for seven days and the highest concentration of ethanol observed was 10.73± 0.045 mg/mL on the 3rd day. Furthermore, this fermentation process exhibited an impressive ethanol yield, with 82.65% achieved by utilizing 17.709 mg/mL of reduced glucose.

The findings of this thesis contribute valuable insights into cellulase-producing bacteria and their potential applications in bioethanol production. This research holds significant implications for developing efficient and sustainable strategies to utilize lignocellulosic biomass, further advancing the use of bioethanol as a green and renewable energy source in the context of a sustainable bio-economy.

CHAPTER 6

CONCLUSION

This study underscores the significant potential of eco-friendly alternatives to fossil fuels, with a particular focus on utilizing bioethanol derived from lignocellulosic biomass. Through the isolation of cellulase-producing bacteria from diverse sources in Nepal, *Bacillus cereus spp.* strain U1C4, obtained from cow dung, demonstrated remarkable cellulolytic activity. The choice of *Saccharum spontaneum* as the optimal carbon source for cellulase production highlights its suitability in driving enzymatic processes. The purification of the cellulase enzyme using a sequence of $(NH4)_2SO_4$ precipitation, dialysis, and gel permeation chromatography yielded impressive results, achieving a substantial recovery rate of 5.4%, a notable 6.34-fold purification, and a high specific activity of 4.693 U/mg. The purified enzyme's molecular weight was determined to be 50 kDa, and its optimal operating conditions were identified at a temperature of 35°C and pH 8, with *Saccharum spontaneum* and peptone serving as the preferred carbon and nitrogen sources, respectively. Upon kinetic analysis, the purified CMCase enzyme showed K_m and V_{max} values of 5.16 mg/mL and 33.22µmol/mL/min, respectively. The successful up-scaling of enzyme production using an Electrolab fermenter 360 demonstrates the feasibility of large-scale cellulase production for potential industrial applications. Notably, ethanol production using *S. cerevisiase* strain CDBT-2 after saccharification of *S. spontaneum* by U1C4 strain resulted in the highest concentration of ethanol, 10.73±0.045 mg/mL on the 3rd day of fermentation. Furthermore, this fermentation process exhibited an impressive ethanol yield, with 82.65% achieved by utilizing 17.709 mg/mL of reduced glucose. Hence this research highlights the enzymatic efficiency in converting complex lignocellulosic biomass into valuable ethanol. Also, the successful isolation, purification, and application of cellulase enzyme underscore the potential for future advancements in lignocellulosic biomass conversion, providing a significant stride towards achieving more sustainable energy practices.

CHAPTER 7

RECOMMENDATION

This research holds great potential for widespread application across various industries, as it offers valuable insights into enhancing yield and process efficiency. Some of the notable areas where its application can lead to significant improvements have been outlined below:

- It can be recommended for further refinement, production optimization, and policy implementation. Notably, the scaling-up of this process to industrial applications is highly encouraged.
- This study's findings can provide valuable recommendations to the Alternative Energy Promotion Centre, Nepal for formulating effective policies and measures to promote bioethanol as a sustainable fuel option.
- This study holds significant potential for municipalities and other institutions engaged in waste management and alternative energy generation centers of Nepal.
- It can be further research to optimize effect of agitation rate on enzyme production.

REFERENCES

Acharya S, Chaudhary A (2011) Effect of nutritional and environmental factors on cellulases activity by thermophilic bacteria isolated from hot spring. J Sci Ind Res 70: 142-148.

Abdullah, R., Tahseen, M., Nisar, K., Kaleem, A., Iqtedar, M., Saleem, F., & Aftab, M. (2021). Statistical optimization of cellulases by Talaromyces thermophilus utilizing Saccharum spontaneum, a novel substrate. *Electronic Journal of Biotechnology*, *51*, 79–87. https://doi.org/10.1016/j.ejbt.2021.03.007

Akhtar, N., Gupta, D., and Goyal, A. (2016). Recent advances in pretreatment technologies for efficient hydrolysis of lignocellulosic biomass. *Environmental Progress & Sustainable Energy.* 35, 489-511. Doi: 10.1002/ep.12257

Amersham Biosciences, Affinity Chromatography; Principles and Methods, Amersham Biosciences, Uppsala, Sweden, 2002.

Andersen, N. (2007). Enzymatic Hydrolysis of Cellulose: Experimental and Modeling studies. *Applied Microbiology*, *4*(1), 39–45.

Arantes, V., & Saddler, J. N. (2011). Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. *Biotechnology for Biofuels*, 4, 1-12. DOI: 10.1186/1754-6834-4-4. (https://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/1754-6834-4-4)

Ariffin, H., Hassan, M. A., Shah, U. K. M., Abdullah, N., Ghazali, F. M., & Shirai, Y. (2008). Production of bacterial endoglucanase from pretreated oil palm empty fruit bunch by bacillus pumilus EB3. *Journal of Bioscience and Bioengineering*, *106*(3), 231–236. https://doi.org/10.1263/jbb.106.231

Arefin, M.A.; Rashid, F.; Islam, A. A Review of Biofuel Production from Floating Aquatic Plants: An Emerging Source of Bio-Renewable Energy. Biofuels Bioprod. Biorefin. 2021, 15, 574-591

Afzal, S., Saleem, m., Yasmin, R. et al. Pre and post cloning characterization of a β-1,4-endoglucanse from Bacillus sp. Molecular Biology Reports. 2010 Apr 37(4): 1717-1723(2010). https://doi.org/10.1007/s11033-009-9592-5

Bischoff, K. M., Rooney, A. P., Li, X. L., Liu, S., & Hughes, S. R. (2006). Purification and characterization of a family 5 endoglucanase from a moderately thermophilic strain of Bacillus licheniformis. *Biotechnology Letters*, *28*(21), 1761–1765. https://doi.org/10.1007/s10529-006-9153-0

Broda, M., Yelle, D. J., & Serwańska, K. (2022). Bioethanol Production from Lignocellulosic Biomass—Challenges and Solutions. *Molecules*, *27*(24). https://doi.org/10.3390/molecules27248717

Bayer, E.A., E. Morag, R. Lamed, 1994. The Cellulosome—a treasure-trove for biotechnology. Trends Biotechnol., 12: 379-86

Britannica, T. Information Architects of Encyclopaedia (2023, July 23). Cellulose. Encyclopedia Britannica. https://www.britannica.com/facts/cellulose

Bušić, A., Mardetko, N., Kundas, S., Morzak, G., Belskaya, H., Šantek, M. I., Komes, D., Novak, S., & Šantek, B. (2018). Bioethanol production from renewable raw materials and its separation and purification: A review. *Food Technology and Biotechnology*, *56*(3), 289–311. https://doi.org/10.17113/ftb.56.03.18.5546

Batubara, U. M., Mardalisa, M., Suparjo, S., Maritsa, H. U., Pujianto, E., & Herlini, M. (2021). Isolation and characterization of cellulolytic bacteria diversity in peatland ecosystem and their cellulolytic activities. *IOP Conference Series: Earth and Environmental Science*, *934*(1), 012028. https://doi.org/10.1088/1755-1315/934/1/012028

Baruah, J., Nath, B. K., Sharma, R., Kumar, S., Deka, R. C., Baruah, D. C., & Kalita, E. (2018). Recent trends in the pretreatment of lignocellulosic biomass for value-added products. *Frontiers in Energy Research*, *6*. https://doi.org/10.3389/fenrg.2018.00141

Coughlan MP (1985). The properties of fungal and bacterial cellulases with comment on their production and application. *Biotechnology and genetic engineering reviews* Newcastle-upon-Tyne: Inter science Russell GE 1985, 3:37-109.

Chang, Y. H., Chang, K. S., Chen, C. Y., Hsu, C. L., Chang, T. C., & Jang, H. Der. (2018). Enhancement of the efficiency of bioethanol production by Saccharomyces cerevisiae via gradually batch-wise and fed-batch increasing the glucose concentration. *Fermentation*, *4*(2). https://doi.org/10.3390/fermentation4020045

Davis, S. C., Parton, W. J., Grosso, S. J. D., Keough, C., Marx, E., Adler, P. R., & DeLucia, E. H. (2012). Impact of second-generation biofuel agriculture on greenhouse-gas emissions in the corn-growing regions of the US. Frontiers in Ecology and the Environment, 10(2), 69–74. https://doi.org/ 10.1890/110003.

Das, P. K., Das, B. P., & Dash, P. (2020). Potentials ofpostharvest rice crop residues as a source of biofuel. Refining Biomass Residues for Sustainable Energy and Bioproducts, 275–301.https://doi.org/10.1016/b978- 0-12-818996-2.00013-2.

Dahadha, S., amin, Z., Bazyar Lakeh, A.A., and Elbeshbishy, E. (2017). Evaluation of different pretreatment processes of lignocellulosic biomass for enhanced biomethane production. *Energy Fuels.* 31, 10335-10347. doi: 10.1021/acs.energyfuels.7b02045

de Souza, W. R. (2013). Microbial degradation of lignocellulosic biomass. *Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization*. https://doi.org/10.5772/54325

Deguchi, S., Tsujii, K. and Horikoshi, K. (2006) Cooking Cellulose in Hot and Com- pressed Water. Chemical Communications, 31, 3293-3295. https://doi.org/10.1039/b605812d

Endo, K., Hakamada, Y., Takizawa, S., Kubota, H., Sumitomo, N., Kobayashi, T., & Ito, S. (2001). A novel alkaline endoglucanase from an alkaliphilic Bacillus isolate: Enzymatic properties, and nucleotide and deduced amino acid sequences. *Applied Microbiology and Biotechnology*, *57*(1–2), 109–116. https://doi.org/10.1007/s002530100744

Eliasson A, Christensson C, Fredrik WC, and Hahn-Hagerda B (2000) Anaerobic Xylose Fermentation by Recombinant *Saccharomyces cerevisiae* Carrying XYL1, XYL2, and XKS1 in Mineral Medium Chemostat Cultures. Applied and Environmental Microbiology. 66(8): 3381-3386.

El Akkari, M., Rechauche`re, O., Bispo, A., Gabrielle, B., & Makowski, D. (2018). A meta-analysis of the greenhouse gas abatement of bioenergy factoring in land use changes. Scientific Reports, 8(1), 1–7.

EIA. (2019). USEPA, biomass explained waste-to-energy (municipal solid waste). Retrieved from: https://www. eia.gov/energyexplained/biomass/waste-to-energy.php

Farhat, W., Venditti, R.A., Hubbe, M., Taha, M., Becquart, F., and Ayoub, A. (2017). A review of water-resistant hemicellulose-based materials: processing and applications. *ChemSusChem*. 10, 305-323. https://doi.org/10.1002/cssc.201601047

Gaur, R., & Tiwari, S. (2015). Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from Bacillus vallismortis RG-07. *BMC Biotechnology*, *15*(1). https://doi.org/10.1186/s12896-015-0129-9

Goldschmidt F (2008) From Cellulose to Ethanol: Engineering Microorganisms to Produce Biofuel. Term Paper submitted in Institute of Bio geochemistry and Pollutant Dynamics, Department Environmental Sciences, ETH Zurich.

Gupta, P., Samant, K., & Sahu, A. (2012). Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. *International Journal of Microbiology*, *2012*. https://doi.org/10.1155/2012/578925

Graves, C., Ebbesen, S.D., Mogensen, M., & Lackner, K.S. (2011). Sustainable hydrocarbon fuels by recycling CO_2 and H_2O with renewable or nuclear energy. *Renewable and Sustainable Energy Reviews*, 15(1), 1-23. https://doi.org/10.1016/j.rser.2010.07.014

H, S., N, Z., S, Z., A, A., B, A., F, I., SU, R., & MH, R. (2021). Microbial Cellulases: A Review on Strain Development, Purification, Characterization and their Industrial Applications. *Journal of Bacteriology and Mycology*, *8*(5). https://doi.org/10.26420/jbacteriolmycol.2021.1180

Hafiz Muhammad Nasir, I., Ishtiaq, A., Muhammad Anjum, Z., & Muhammad, I. (2011). Purification and characterization of the kinetic parameters of cellulase produced from wheat straw by Trichoderma viride under SSF and its detergent compatibility. In *Advances in Bioscience and Biotechnology* (Vol. 2011).

Hakeem, K. R., Jawaid, M., & Alothman, O. Y. (2015). Agricultural biomass based potential materials. *Agricultural Biomass Based Potential Materials*, *April*, 1–505. https://doi.org/10.1007/978-3-319-13847-3

Han, Y. W., Kim, Y. W., & Shin, H. D. (1995). Cellulase production of Bacillus species. Journal of Microbiology and Biotechnology, 5(1), 11-15.

Illumina. (2013). 16S Metagenomic Sequencing Library. *Illumina.Com*, *B*, 1–28. http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

Isikgor, F. H., & Becer, C. R. (2015). Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. *Polymer Chemistry*, *6*(25), 4497–4559. https://doi.org/10.1039/c5py00263j

Jedvert, k., and Heinze, T. (2017). Cellulose modification and shaping- a review. J Polym. Eng. 37, 845-860. https://doi.org/10.1515/polyeng-2016-0272

Joshi, J., Dhungana, P., Prajapati, B., Maharjan, R., Poudyal, P., Yadav, M., Mainali, M., Yadav, A. P., Bhattarai, T., & Sreerama, L. (2019). Enhancement of ethanol production in electrochemical cell by saccharomyces cerevisiae (CDBT2) and Wickerhamomyces Anomalus (CDBT7). *Frontiers in Energy Research*, *7*(7), 1-11 https://doi.org/10.3389/fenrg. 2 019.00070.

Joshi, C. P., Thakur, V. V., & Kumar, R. (2011). Bioenergy: A sustainable and economically competitive option for climate mitigation and food security. Energy and Environment, 22(5), 491-512.

Krishna C. Duong-Ly, Sandra B. Gabelli, Chapter Seven- Salting out of Proteins Using Ammonium Sulfate Precipitation, Editors(s): Jon Lorsch, *Methods in Enzymology*, Academic Press Volume 541, 2014, Pages 85-94, ISSN 0076-6879, https://doi.org/10.1016/B978-0-12-420119-4.00007-0.

Kumar, D., Ashfaque, M., Muthukumar, M., Singh, M., & Garg, N. (2012). Production and characterization of carboxymethyl cellulase from Paenibacillus polymyxa using mango peel as substrate. *Journal of Environmental Biology*, *33*(1), 81–84.

Kumar, R., Sharma, R.K, and Singh, A.P. (2017). Cellulose based grafted biosorbents-Journey from lignocellulose biomass to toxic metal ions sorption applications- a review. *Journal of Molecular Liquids* .232, 62-93. https://doi.org/10.1016/j.molliq.2017.02.050

Lam, K. C., Ariffin, H., & Hassan, M. A. (1998). Non-pathogenic nature of Bacillus species: A review. Journal of Applied Microbiology, 85(3), 381-386.

Lee Y-J, Kim B-K, Lee B-H, Jo K-I, Lee N-K, Chung C-H, Lee Y-C and Lee J-W (2008) Purification and characterization of cellulase produced by *Bacillus amyloliquefaciens* DL-3 utilizing rice hull. *Bioresour Technol*. 99(2): 378-386

Lee RL, Paul JW, Willem HV, Isak SP (2002). Microbial Cellulose Utilization: Fundamentals and Biotechnology. Microbiology and Molecular Biology Reviews, 66 (3): 506-577.

L. J. Cseke, P. B. Kaufman, G. K. Podila, and C.-J. Tsai, Handbook of Molecular and Cellular Methods in Biology and Medicine, CRC Press, Boca Raton, Fla, USA, 2nd edition, 2004.

Li W, Huan X, zhou Y, Ma Q and Chen Y (2009) Simultaneous cloning and expression of two cellulases genes from *Bacillus subtilis* newly isolated from Golden Takin (Budorcas taxicolor Bedfordi). *Biochem Biophys Res Commun.* 383(4): 397-400

Lugani, Y., Singla, R., & Singh Sooch, B. (2015). Optimization of cellulase production from newly isolated bacillus sp.. Y3. *Journal of Bioprocessing & Biotechniques*, *5*(11). https://doi.org/10.4172/2155-9821.1000264

Lynd, LR, Weimer PJ, Van Zyl WH. And Pretorius IS, (2002). Microbial Cellulose Utilization: fundamentals and biotechnology. Microbiol. Mol. Biol. Rev., 66: 506-577

Marszałek, J., & Kamiński, W. (2008). Environmental Impact of Bioethanol Production Wpływ Produkcji Bioetanolu Na Środowisko. *Proceedings of ECOpole*, *2*(1).

Malherbe, S., Services, B., & Cloete, T.E. (2002). Lignocellulose biodegradation: Fundamentals and applications. Re/views in Environmental Science & Bio/Technology, June 2015, 105-114. https://doi.org/10.1023/A

Msangi, Siwa & Sulser, Timothy & Rosegrant, Mark & Valmonte-Santos, Rowena, 2007. "**Global Scenarios for Biofuels: Impacts And Implications For Food Security And Water Use**," Conference papers 331581, Purdue University, Center for Global Trade Analysis, Global Trade Analysis Project.

Nowakowski, A. B., Wobig, W. J., & Petering, D. H. (2014). Native SDS-PAGE: High resolution electrophoretic separation of proteins with retention of native properties including bound metal ions. Metallomics, 6(5), 1068–1078. https://doi.org/10.1039/c4mt00033a

NREL. Determination of Structural Carbohydrates and Lignin in Biomass Laboratory Analytical Procedure (LAP). NREL/TP. 2012; 510-42618

Pandey, Diwakar. (2014). Re: How does one convert released reduction sugars (mg/mL) to enzyme activity (IU)? Retrieved from: https://www.researchgate.net/post/How_does_one_convert_released_reduction_sugars_mg_ml_to_enzyme_activity_IU/53c8ffe2cf57d77f6f8b46b2/citation/download.

Pirolini, A. (2015). What is Bioethanol? *Animal Genetics*, *39*(5), 561–563.http://www.esru.strath.ac.uk/EandE/Web_sites/02-03/biofuels/what_bioethanol.htm%0Ahttp://www.esru.strath.ac.uk/EandE/Web_sites/02-03/biofuels/what_bioethanol.htm%0Ahttps://www.azocleantech.com/article.aspx?ArticleID=403

Rashid, H., Rahman, N. A., Nik Him, N. R., & Mokhtar, M. N. (2011). Bioethanol: A review on its production, application, and potential. Renewable and Sustainable Energy Reviews, 15(9), 4022-4031.

R. Kataria and S. Ghosh (2011). Saccharification of Kans grass using enzyme mixture from *Trichoderma reesei* for bioethanol production. *Bioresource Technology* 2011; 102: 9970–9975.

Rosenberg, Ian M., 2013. Protein analysis and purification: benchtop techniques. 141. Springer Sciences & Business Media

Singh, A., 1999. Engineering enzyme properties. Ind. J. Microbiol., 39: 65-77https://www.addgene.org/protocols/create-glycerol-stock

Singh, R., Kumar, M., Mittal, A., & Mehta, P.K. (2016). Micrbial cellulases in Industrial applications. *Annals of Applied Bio-Sciences, 3.*

Singh S, Bajaj BK. Bioprocess optimization for production of thermo alkali-stable protease from *Bacillus subtilis* K-1 under solid-state fermentation. *Prep Biochem Biotechnol.* 2016 Oct 2; 46(7):717-24. doi: 10.1080/10826068.2015.1135455.PMID: 26760481

Siddiqui KS, Shemsi AM, Anwar MA, Rashid MH, Rajoka MI (1999). Partial and complete alteration of surface charges of carboxymethylcellulase by chemical modification: themostabilization in water-miscible organic solvent. Enzyme Microb. Technol. 24: 599-608.ISS0141-0229. https://doi.org/10.1016/S0141-0229 (98)00170-7

Shankar, T., & Isaiarasu, L. (2011). Cellulase Production by Bacillus pumilus EWBCM1 under Varying Cultural Conditions. *Middle-East Journal of Scientific Research*, *8*(1), 40–45.

Sharma, D., Joshi, B., Bhatt, M. R., Joshi, J., Malla, R., Bhattarai, T., & Sreerama, L. (2015). Isolation of cellulolytic organisms from the gut contents of termites native to Nepal and their utility in saccharification and fermentation of lignocellulosic biomass. *Journal of Biomass to Biofuel*. https://doi.org/10.11159/jbb.2015.002

Sharada, R., G. Venkateswarlu, S. VenKateshwar and S. AnandRao, 2013. Production of cellulases. A review. IJPCBS 3(4): 1070-1090.

Suali, E., & Suali, L. (2023). Impact assessment of global biofuel regulations and policies on biodiversity. In *Environmental Sustainability of Biofuels*. Elsevier Inc. https://doi.org/10.1016/b978-0-323-91159-7.00012-6

Sulyman, A. O., Igunnu, A., & Malomo, S. O. (2020). Isolation, purification and characterization of cellulase produced by Aspergillus niger cultured on Arachis hypogaea shells. *Heliyon*, *6*(12). https://doi.org/10.1016/j.heliyon.2020.e05668

Tan, S. C., & Yiap, B. C. (2009). DNA, RNA, and protein extraction: The past and the present. *Journal of Biomedicine and Biotechnology*, *2009*. https://doi.org/10.1155/2009/574398

Thomas, A., Laxmi, M., & Benny, A. (2021). Bioethanol Production of Cellulase Producing Bacteria from Soils of Agrowaste Field. *Journal of Scientific Research*, *13*(2), 643–655. https://doi.org/10.3329/jsr.v13i2.50574

Thomas, L. (2019). Protein Purification Techniques. News-Medical. https://www.newsmedical.net/life-sciences/Protein-Purification-Techniques.aspx

Tse, T. J., Wiens, D. J., & Reaney, M. J. T. (2021). Production of bioethanol—a review of factors affecting ethanol yield. *Fermentation*, *7*(4), 1–18. https://doi.org/10.3390/fermentation7040268

Tsai, W.-T. (2019). An analysis of operational efficiencies in the waste-to-energy (wte) plants ofKaohsiung Municipality (Taiwan). Resources, 8(3), 125. https://doi.org/10.3390/resources8030125.

U.S. Department of Energy, Alternative Fuels Data Center, (2020). https://afdc.energy.gov/data/.

Viera, H., Renata, N., & Jana, Š. (2019). Production of Fuel Ethanol from Lignocellulosic Biomass. *30th International Conference on Wood Science and Technology, ICWST 2019 and 70th Anniversary of Drvna Industrija Journal: Implementation of Wood Science in Woodworking Sector, Proceedings*, 69–76.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology, 173(2), 697–703. https://doi.org/10.1128/jb.173.2.697-703.1991

Wijayanti, S. D., Oliviani, K., Kusnadi, J., & Putri, R. A. C. (2020). Characterization of crude cellulase enzyme produced by Bacillus licheniformis P12 isolate. *IOP Conference Series: Earth and Environmental Science*, *475*(1). https://doi.org/10.1088/1755-1315/475/1/012085

William, S., Feil, H., & Copeland, A. (2004). Bacterial DNA Isolation CTAB Protocol Bacterial genomic DNA isolation using CTAB Materials & Reagents. *Doe Joint Genome Institute*, 4. http://my.jgi.doe.gov/general/protocols/JGI-Bacterial-DNA-isolation-CTAB-Protocol-2012.pdf

Yang B, Boussaid A, Mansfield SD (2002). Fast and efficient alkaline peroxide treatment to enhance the enzymatic digestibility of steam exploded soft wood substrate. Biotechnol. Bioeng. 77: 678-684.

Yamada, T., & Fujii, N. (2010). Global warming and acid rain: A new meaning of renewable energy system. Energy Procedia, 5, 1558-1562.

Zhou, X., Li, W., Mabon, R., and Broadbelt, L.J. (2017). A critical review on hemicellulose pyrolysis. Energy Technol. 5, 52-79. https://doi.org/10.1002/ente.201600327

Zabed, H.; Sahu, J.N; Suely, A.; Boyce, A.N.; Farug, G. Bioethanol Production from Renewable Sources: Current Perspectives and Technological Progress. Renew. Sustain. Energy Rev. 2017, 71, 475-501

Zhang M, Eddy C, Deanda K, Finkelstein M, Picataggio S, (1995) Metabolic Engineering of a Pentose Phosphate Pathway in Ethanologenic *Zygomonas mobilis.* Science 267: 240-243. https://doi.org: 10.1126/science.267.5195.240. PMID: 17791346

Zheng, C. (2018). Growth characteristics and enzyme production optimization of lipase Producing Strain. IOP Conference Series: Earth and Environmental Science, 108(4). https://doi.org/10.1088/1755-1315/108/4/042087

APPENDICES
Appendix I

Media compositions and preparations of reagents and buffers

(A) Media

CMC media

Components	g/L
K_2HPO_4	0.2
KH_2PO_4	0.2
$MgSO_4$	0.2
NaCl	0.2
$NaNO_3$	1
$CaCO_3$	0.01
Yeast extract	0.5
CMC	10
Agar	15
pH	7

Congo- Red Agar Media

Components	g/L
KH_2PO_4	0.5
$MgSO_4$	0.25
CM cellulose	2
Agar	15
Congo red	0.2
Gelatin	2
Distilled Water	1
pH	6.8-7.2

Nutrient Agar

Ingredients	g/L
Peptic digest of animal tissue	5
Beef extract	1.50
Yeast extract	1.50
Sodium Chloride	5
Agar	15
pH	7.4±0.2

Nutrient Broth

Ingredients	g/L
Peptic digest of animal tissue	5
Beef extract	1.50
Yeast extract	1.50
Sodium Chloride	5
pH	7.4±0.2

Peptone, yeast Extract and Nutrient (PYN) Media

Composition	g/L
Peptone	3.5
Yeast extract	3
KH_2PO_4	2
$MgSO_4$	1
$(NH4)_2.\ SO_4$	1
Glucose (5%)	12.5

Luria Bertani Broth/Agar

Ingredients	g/L
Bacto Tryptone	10
Bacto Yeast extracts	5
NaCl	5

DNS reagent preparation

1) Solution I: Dissolve 75 g Sodium potassium tartarate in 125 mL d/w
2) Soution II: Dissolve 4 g of NaOH in 50 mL distilled water and add 2.5g DNS.
3) Mix solution I and solution II and maintain volume of 250 mL. After mixing above ingredients, the solution was kept in a dark brown bottle or should be wrapped properly to avoid photo oxidation.

Bradford Reagent Preparation/ Coomassie Brilliant Blue G250 preparation

1) Weigh 0.1 g CBB-G250 dye and add 50 ml 95% ethanol.
2) Slowly and carefully, add 100 mL 85% phosphoric acid.
3) Mix until the blue dye is completely dissolved.
4) Add the dye/ethanol/Phosphoric acid solution to 850 mL pure sterile water.
5) Filter any precipitates.
6) Store Bradford Reagent at 4^oC for months.

Preparation of Congo red solution

1) 0.1g Congo red was weighed and dissolved in few ml of dissolved water in a 100 mL reagent bottle.
2) The volume was adjusted to 100 ml by adding distilled water after complete dissolving of Congo red.

Preparation of Phosphate buffer (pH 7, 0.05 M)

1) Prepare 160 mL of distilled water in a suitable container.
2) Add 1.549 g of Sodium Potassium Dibasic Heptahydrate to the solution.
3) Add 0.582 g of Sodium Potassium Monobasic Monohydrate to the solution.
4) Adjust solution to final desired pH using HCL or NaOH.
5) Add distilled water until the volume is 200 mL.

Preparation of Citrate buffer (pH 4.8, 0.05 M)

1) Prepare 160 ml of distilled water in a suitable container
2) Add 1.566g of Sodium Citrate dihydrate to the solution.
3) Add 0.897g of Citric acid to the solution.
4) Adjust solution to final desired pH using HCl or NaOH.
5) Add distilled water until the volume is 200 mL.

Iodine solution preparation

1) Dissolve 10 g of Potassium iodide (KI) in about 20-30 ml of distilled water.
2) Add iodine and heat gently with constant mixing until iodine is dissolved.
3) Dilute to 100 mL with distilled water.
4) Store in amber-glass-stoppered bottle in the dark.

Iodoform test

-10 drops of distillate and 25 drops of iodine along with 10 drops of NaoH were added in the test tube.

-After few minutes, cloudy formation, yellow precipitate and antiseptic smell confirms the presence of bioethanol in the test tubes.

Reagents for SDS-PAGE

1) **30% Acrylamide solution (29:1) (for 100 mL)**

	Weight
Acrylamide, C3H5NO (MW: 71.08)	29g
Bis- Acrylamide (MW: 154.17)	1
TDW	up to 100 mL

Note: Dark condition should be maintained
And stored in room temperature.

2) **Lower Tris (pH 8.8) (for 50 mL)**

1.5 M Tris (Tris base)	9.0855 g
TDW	up to 50 mL

Note: Should be autoclaved and stored in 4°C

3) **Upper Tris (pH 6.8) (for 50 mL)**

0.5 M Tris (Tris base)	3.0285g
TDW	up to 50 mL

Note: Should be autoclaved and stored in $4\ ^{O}$C

4) **10% Ammonium per sulphate $(NH_4)2S_2O_8$ (MW: 228.2)**

APS	0.1 g
TDW	up to 1 mL

Note: Dark condition should be maintained and should be freshly
prepared and stored at $-20\ ^{O}$C

5) **Loading dye/ Sample buffer/Loading buffer**

	For 10 mL (pH 6.8)
Upper Tris	1.25 mL
10% SDS	3 .0 mL
Glycerol	4.75mL
B-mercaptoethanol	0.5 mL
0.1% Bromophenol Blue dye (0.01 g in 10 mL)	0.5 mL

Note: Above all five components should be mixed properly

in the following order, dark condition should be maintained,
and stored in 4°C

6) **Comassie Brilliant Blue (CBB) staining solution (For 250 mL)**

CBB G-250	0.25g
Glacial acetic acid	12.5 ml
Methanol	125 ml
TDW	up to 112.5 ml

Note: Dark condition should be maintained and stored in room temperature

De-stain solution (for 250 mL)

Glacial acetic acid	18.75 ml
Methanol	12.5 ml
TDW	up to 218.75 ml

Note: Stored in room temperature

7) **Running/ Electrolysis buffer , pH 8.4 (for 1000 mL)**

39 mM Tris	4.724 g
48 mM Glycine	3.603 g
0.1% SDS	0.37 g
TDW	up to 1000 ml

Note: Should be freshly prepared and stored in room temperature

Appendix II: Standard curves

1. Glucose standard graph

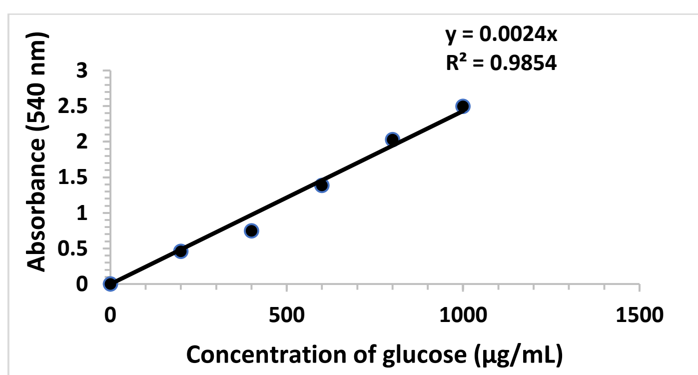


Figure 29: Calibration curve of glucose for reducing sugar estimation

2. Protein standard graph

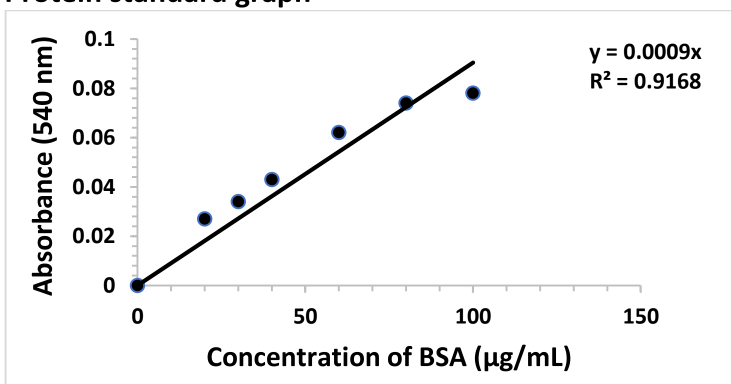


Figure30: Calibration curve of BSA for Bradford assay (Protein estimation)

3. Standard graph of Ethanol

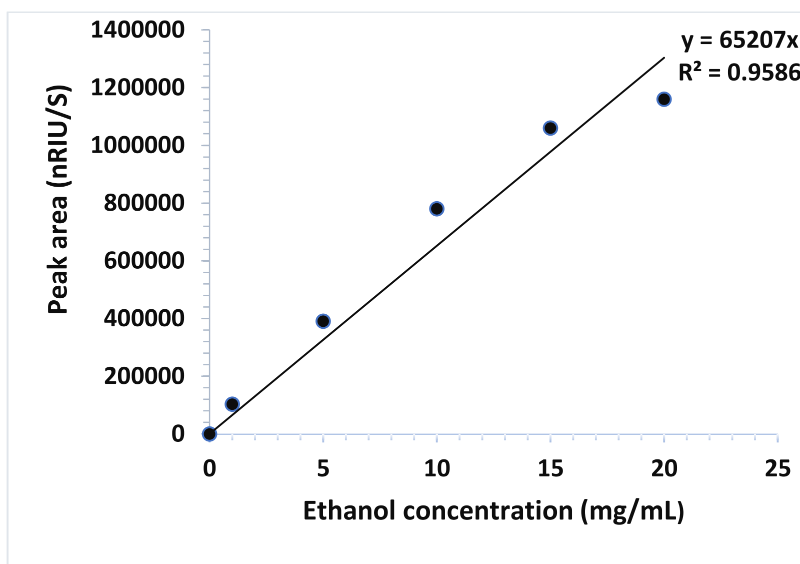


Figure 31: Standard curve of absolute ethanol for estimation of bioethanol concentration by HPLC.

Appendix III: Sequence of Isolate U1C4

Sequencing of PCR products using Sanger Sequencer and electropherogram image

The sequencing was carried out at both directions using forward and reverse primers in ABI310 Genetic Analyzer. The raw sequences were quality trimmed. And the sequences with both forward and reverse reads were aligned into a consensus sequence.

The final aligned and merged sequence:

> UIC4

CGCAATGGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGG
GTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTT
GACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCT
TAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAG
ACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAG
ATATGGAGGAACACCAGTGGCG

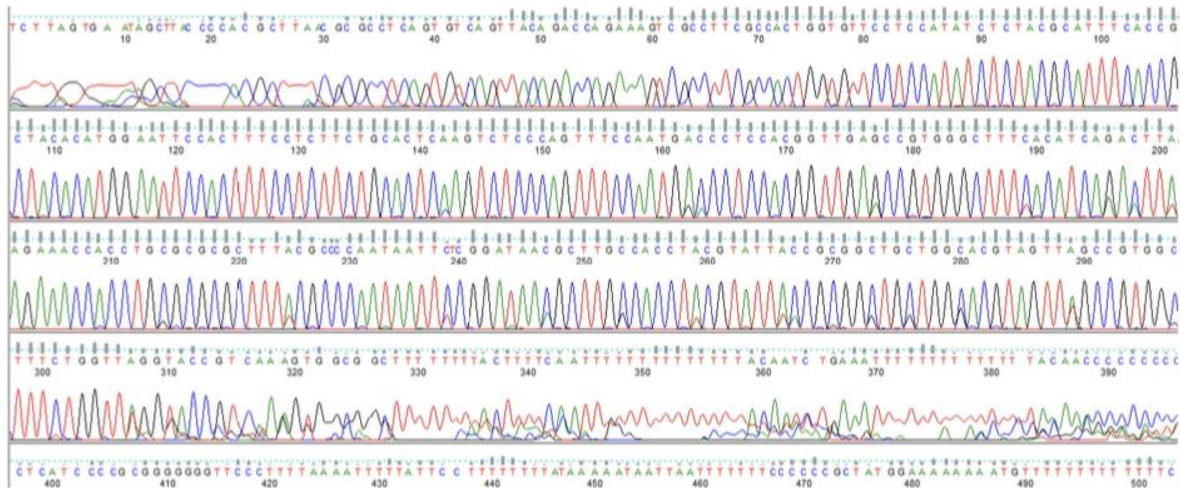


Figure 32: Electropherogram image of the U1C4 isolate

Appendix IV: Table

Table 1: The amount of the solid ammonium sulfate required for a solution to give desired final saturation at 0 ^{O}C (Harris, 2001).

Initial concentration of ammonium sulfate	Percentage saturation at 0°																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (grams) to be added to 1 liter of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

Appendix V

Photographs during thesis work

Figure 1: Gel permeation chromatography during enzyme purification process

Figure 2: Iodoform test for the estimation of bioethanol

Figure 3: Cow-dung sample collection farm house at Chovar, Kirtipur, Nepal

Figure 4: Qualitative cellulolytic activity test (Well diffusion method) of five different substrate by U1C4 cellulolytic isolate

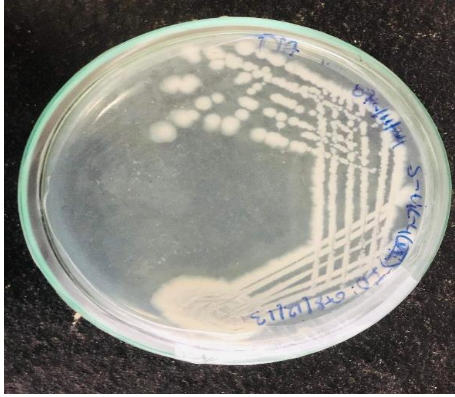


Figure 5: Pure colony of U1C4 isolate .

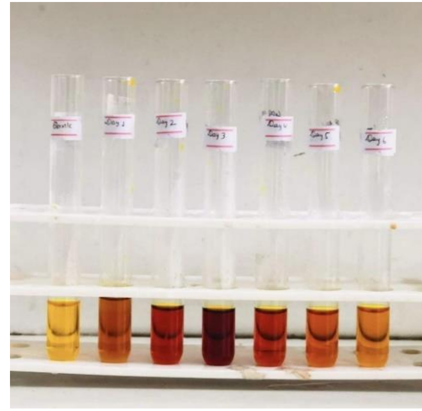


Figure 6: Reducing sugar test by DNS Method.

FIgure 7: SDS-PAGE gel run of different purification stages of cellulase enzyme produced by U1C4 isolate.

Figure 8: *Saccharum spontaneum* (Kans grass) grown wildly on CDBT ground, T.U, Kirtipur, Nepal.

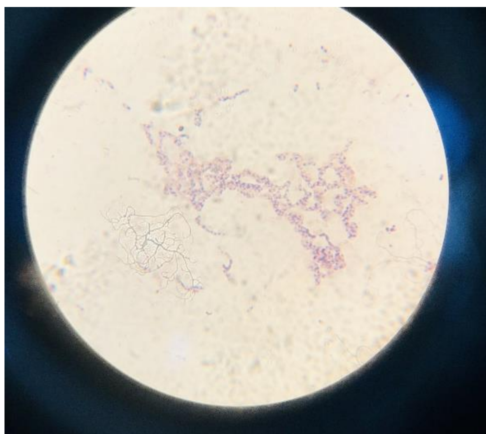


Figure 9: Grams staining of U1C4 isolate.

Figure 10: Identification of compound by using HPLC.