Cellulase production from green macroalgal biomass *Ulva* sp. using cellulolytic bacteria

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By

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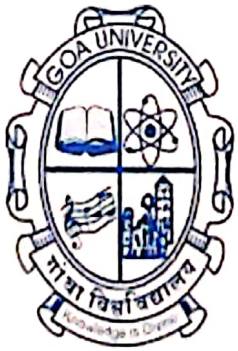

GOA UNIVERSITY

APRIL 2023

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I hereby declare that the data presented in this Dissertation report entitled, "Cellulase production from green macroalgal biomass *Ulva* sp. using cellulolytic bacteria" is based on the results of investigations carried out by me in the Marine biotechnology discipline at the School of Biological Sciences and Biotechnology, Goa University under the Guidance of Dr. Meghanath Prabhu and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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Chapter 1: Introduction & Literature Review

The most prevalent biomass on earth is cellulose. They make up the majority of plant cell walls and the most prevalent renewable bioresource produced in the biosphere (Jarvis, 2003). A homopolymer of d-glucose units connected by β-1,4 links makes up cellulose.

Cellulose being the most abundant organic compound on earth, is an important structural component of the primary cell wall of green plants (Siddhanta et al., 2013).

A variety of algae, particularly marine macroalgae and oomycetes also contain it. Since it has a number of advantages over cellulose from land-based biomass, interest of cellulose from macroalgae has increased recently. Macroalgae have purer fractions that would be more appropriate for biomedical applications than lignocellulose. Furthermore, the absence of lignin enables extraction of cellulose under softer conditions, resulting in less damaged portions of cellulose. Macroalgae are a quick growing, inexpensive source of biomass that may be farmed without a lot of water or arable land.

Additionally, because macroalgae can be grown without the use of fertilisers, eutrophication along the coast is avoided. It has been effective to extract cellulose from red, green and brown macroalgae. The gross amount of cellulose varies widely across different species, typically falling between 1 and 15% (w/w). The maturity of the biomass has a significant impact on the seasonal variations in the cellulose concentration. The genus Ulva includes a group of cellulose-containing green macroalgae. It is a macroalgae from the Chlorophyta family of green algae that is cultivated for commercial use. Ulva is widely distributed along the ocean's coastline and is a cheap and abundant source of biomass. Ulva's high productivity and steadfast ability to thrive in a variety of worldwide growing environments make it particularly well suited for sea-based biomass production (Dominguez & Loret, 2019).

Due to its superior nutritional content and high concentrations of polysaccharides, proteins, vitamins, and trace minerals, ulva is one of the most extensively consumed edible seaweeds in the world (Morelli, 2011). Ulva's cell walls are made of the polysaccharide ulvan, which acts as a structural component, and cellulose.(Wahlström et al., 2020).

Appropriate procedures for the efficient treatment and use of wastes containing cellulose as inexpensive carbon sources have thus been of great commercial interest (Shanmugapriya, 2012). The dry weight of secondary sources of biomass, such as agricultural wastes, and 50%

of the dry weight of plant biomass are both made up of cellulose. Recent years have seen an increase in the environmental build-up of cellulosic waste from industry and agriculture (Lü et al., 2011). The ability of cellulose to be converted into bio-based products and bioenergy has attracted interest on a global scale. It has been found, that the most significant renewable resource for bioconversion is celluloses, it has been found (Maki et al., 2009). It has become economically advantageous to create a technology for efficiently hydrolysing cellulosic biomass (Patagundi et al., 2014). The challenge in this regard is to create procedures that are commercially viable. Three different types of enzymes- cellobiohydrolase, endoglucanase or carboxymethylcellulase (CMCase), and beta-glucosidases- must work in concert for the enzyme to be completely hydrolysed.

There are still a lot of natural cellulosic sources, as well as a lot of cellulose containing raw materials and waste products, that are not being used to their full potential or could be used more effectively. This is true despite the fact that these resources are used widely and extensively (Bhat, 2000).

Cellulose is often destroyed by cellulase enzyme. Several microbes, primarily bacteria and fungus, generate this enzyme (Patagundi et al., 2014). The possibility for using bacteria that grow more quickly than fungus in the synthesis of cellulase is high. Cellulase production with bacteria is not a common practice, though. Some bacterial taxa, including *Cellulomonas*, *Cellvibrio*, Pseudomonas sp. (Nakamura, 1982) *Bacillus*, and *Micrococcus* (Immanuel, 2006) have also been shown to possess cellulolytic properties. In microorganisms, the production of enzymes is tightly regulated, but these constraints can be relaxed to increase productivity.

It is effective to obtain microbial cellulases using LCB feedstock as a substrate since they can satisfy a lot of industrial demand. To scale up cellulase production, however, for better industrial use of the strain, more research is necessary. The study's next directions could include finding novel strains with the capacity to produce multiple enzymes and further refining pre-treatment techniques for the efficient use of lignocellulosic feedstocks.

Commercial cellulase enzymes are derived from fungi, and adding supplemental enzymatic activity to the fungi's enzymes can increase hydrolysis yield and, as a result, enable low-cost hydrolysis (Ferreira et al., 2018).

Cellulase yields appear to be influenced by a complicated interaction between a number of variables, including the size of the inoculums, pH level, temperature, the presence of inducers, medium additions, aeration, growth time, and other parameters (Immanuel, 2006). Due to the

high expense of their utilisation methods, huge amounts of agricultural, industrial and municipal cellulosic wastes have been building up or being utilised inefficiently. The development of methods for the efficient treatment and utilisation of cellulosic wastes as cheap carbon sources has thus been of substantial commercial interest. The enzyme cellulase breaks down the beta 1,4 glycosidic bonds in the polymer to release glucose molecules (Lee et al., 2008).

Examples of applications for this cellulose-degrading enzyme include the production of washing powders, the extraction of fruit and vegetable juices, and the processing of starches (Camassola & Dillon, 2007). A significant number of bacteria produce cellulase. Either they are extracellular or they are cell-bound. Many microorganisms have the ability to break down cellulose, but only a few numbers of them can create enough free enzymes to completely hydrolyse crystalline cellulose (koomnok, 2006). Cellulases are used in a wide range of industries, including the pulp and paper sector for better drainage, the textile sector for softening cotton and finishing denim, the textile detergent sector for cleaning and colour care, the food sector for mashing, and even the pharmaceutical sector.

In summary, the demand for more stable, highly active, and specialised enzymes will increase quickly alongside the widespread usage of cellulose enzymes in many industrial applications. Therefore, the most intriguing technology of the future will be cellulose enzyme. The technoeconomic viability of various components of cellulose production (such as price, substrate specificity, and specific activity) is another something that needs more research. The present work was carried out to extract cellulose from green seaweed and use it as a carbon source in fermentation process for the production of cellulase.

1.1- Aim and Objectives

The main focus of this study was to use seaweed cellulose as an alternative substrate for production of cellulase enzyme with the following objectives:

1) Collection and identification of seaweeds.
2) Extraction and characterization of seaweed cellulose.
3) Isolation, purification and identification of cellulase producing bacteria.
4) Production of cellulase by bacteria using seaweed cellulose as substrate.
5) Purification of cellulase enzyme.

1.2- Gaps in Research

The demand for more stable, highly active, and specialised enzymes will increase quickly alongside the widespread usage of cellulose enzymes in many industrial applications. Despite the massive and widespread usage of natural cellulosic sources, there are still vast amounts of untapped or underutilized cellulosic sources, cellulose containing raw materials. It has become economically advantageous to create a technology for efficiently hydrolysing cellulosic biomass. Therefore, the most intriguing technology of the future will be cellulase. The technoeconomic viability of various components of cellulose production (such as price, substrate specificity, and specific activity) is another something that needs more research. From the literature review it was visible that seaweed cellulose has not been utilised for the production of high value product cellulase for the commercial applications. The present work was carried out to extract cellulose from green seaweed and use it as a carbon source in fermentation process by bacteria for the production of cellulase.

Chapter 2: Methods and Materials

2.1 Collection and identification of seaweeds

2.1.1Collection of seaweed

Green seaweed was collected from Vagator beach (latitude - 15.600744° longitude - 73.733588 °) located in North Goa region in Goa state on 23 September 2022.

2.1.2 Identification and characterization of seaweed

i- Morphological

Descriptions were used to make species identifications based on morphological characteristics, including attachment, blade shape and size, nature of the margin, cell shape and size in surface view.

ii- Elemental analysis (CHNS%)

After being dried, the seaweed was ground into a fine powder with a mortar and pestle. This fine powder was utilised to determine the CHNS% present in seaweed. The VarioMicro CHNS cube element analyser was used to analyse CHNS. Sulphanilamide was used as the analytical standard for a sample of 2.86 mg.

2.2 Extraction and characterisation of seaweed cellulose

2.2.1 Extraction

To remove the sand, salts, and other impurities present, freshly gathered seaweed was extensively rinsed with tap water and then washed with distilled water. After that, it was submerged in ethanol that was over 99% pure and shaken all night long. Two additional times were spent doing this. Following that, it was centrifuged for 10 minutes at 5000 rpm. After discarding the supernatant, the pellet was suspended in 4% H2O2 and heated for 16 hours at 80°C. Centrifugation was carried out for 15 minutes at 5000 rpm after it had warmed to room temperature. The supernatant was once more discarded. The insoluble

portion was repeatedly washed with water until the pH of the washing water reached 7. The insoluble fraction was washed, then suspended in 5% v/v HCl, heated to 100 C, cooled to room temperature, and then stored for 16 hours. The supernatant was discarded after 15 minutes of centrifuging again at 5000 rpm. With distilled water, the insoluble portion was washed until the pH of the water reached 7. Following that, it was gathered, freeze-dried, and ground into a fine powder using a mortar and pestle (Wahlström et al., 2020).

2.2.2 Cellulose characterization

i- FTIR

FTIR Prestige 21 by Shimadzu was used to obtain FTIR spectra between 4000-350 cm^{-1} with KBr as detector at room temperature for the dry powder of cellulose fraction obtained.

ii- X-Ray Diffraction

The obtained cellulose dry powder was used to analyse the crystal structure and crystallinity index of the cellulose samples at room temperature using Bruker D8 Advance XRD in the range of $2\Theta = 10^{\circ} - 40^{\circ}$ with a scan rate of 3° min^{-1}. The crystallinity index (CrI %) was calculated by using eq.

$$CrI\% = (I_{200} - I_{am}) / I_{200} * 100$$

where I_{am} is the intensity at the local minimum of the curve at $2\Theta = 18^{\circ}$ corresponding to the amorphous sections of the cellulose, and I_{200} is the intensity of the crystalline peak at $2\Theta = 22^{\circ}$ corresponding to the (200) plane (segal, 1959).

iii- Element Analysis (CHNS%)

The obtained cellulose dry powder was characterized for the CHNS% CHNS was analysed as mentioned above in section 2.1.2.(i).

2.3 Isolation, purification and identification of bacterial isolates

2.3.1 Isolation

i- Sample collection, serial dilution, spread plating

In order to isolate bacteria producing cellulase, garden soil sample was collected and from BITS Pilani Goa Campus and then it was serially diluted and dilutions of 10^{-5}, 10^{-6}, and 10^{-7} were spread plated on CMC agar plates. The plates were allowed to incubate at 37°C for 24 hours.

2.3.2 Purification and Screening for cellulase activity

The bacterial colonies were picked up and streaked on nutrient agar to obtain isolated and pure colonies. The plates were submerged with an aqueous solution of 0.1% Congo red for fifteen minutes before being washed with 1 M NaCl to observe the hydrolysis zone (Apun et al., 2000). On CMC (by SRL) agar, the diameter of the clear zone enclosing colonies was measured to represent the organism's cellulolytic activity. The colonies which show maximum zones were selected. The cellulolytic activity of the chosen bacterial isolate in liquid media was also assessed using a more quantitative test method. By calculating the amount of reducing sugars released using the DNS approach, the cellulolytic activity of each culture was determined. The bacterial isolate showing highest cellulolytic activity was chosen to produce cellulase.

2.3.2 Bacterial identification

- Gram staining

  Morphological evaluation and multiple biochemical characterizations were employed to possibly classify the bacterial isolates. The parameters included Gram staining, endospore formation etc. On the basis of Gam staining results the isolates were compared with the Bergey's manual (Wahlström et al., 2020) and were further examined.

- Biochemical characterisation

  Various biochemical test including catalase, endospore staining, strict anaerobe, citrate, VP, methyl red and motility test were conducted.

- Molecular identification

  The strain which shows maximum cellulase activity was further subjected to molecular identification by analysing 16S rRNA sequence. The 27F & 1492R primers amplified a fragment of the 16S rDNA gene. Following PCR, the amplicon was resolved on an agarose gel and filtered to remove impurities. Using forward primers and reverse primers, a PCR amplicon was subjected to a forward and reverse DNA sequencing experiment using Sanger sequencing. The consensus sequence of the 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA partial sequence was used in BLAST searches in NCBI gene bank database. The top 10 sequences were selected based on their highest identity score, and CLUSTAL W, a multiple alignment programme was used to align them. Inferring the evolutionary history from the neighbour joining model. A bootstrap consensus tree created from 1000 replications depicted the evolutionary history of the taxa under study.

2.4 Production of cellulase by bacteria using seaweed as substrate

2.4.1 Production media

Production medium contained (g/100ml) 0.1g of $NH_4H_2PO_4$, 0.02g of KCl, 0.1g $MgSO_4.7H_2O$, yeast extract, 0.5g of cellulose extracted from green seaweed in a 100 mL conical flask. After cooling, the flasks were inoculated with an overnight-grown bacterial culture after being autoclaved at 121°C for 15 minutes to sterilise them. For 24 hours, the inoculated medium was incubated at 37°C. The culture media was centrifuged at 5000 rpm for 15 minutes to extract the crude extract, which was used as an enzyme source, at the conclusion of the fermentation phase.

2.4.2 Enzyme assay for evaluating enzyme activity

To check the cellulase activity miller method was used. In a nutshell, for 30 minutes at 37°C in a shaking water bath, a reaction mixture containing 0.2 mL of crude enzyme solution and 1.8 mL of 0.5% carboxymethyl cellulose (CMC) in 50mM sodium phosphate buffer (pH 7) was incubated. The addition of 2 ml of DNS reagent stopped the process. For the development of colour, it was then held in a boiling water bath for 10 minutes. At 540 nm, the OD of the samples was evaluated in comparison to a blank that contained all the reagents but no crude enzyme. Standard of glucose with concentration of 0.2mg/ml was prepared and CMC was used as substrate control and then their OD was taken at 540 nm using UV-1800 by Shimadzu UV Spectrophotometer (Miller, 1959). The enzyme activity was are as units per gram of medium (U/ml) or µmol/min).

2.4.3 Enzyme activity

One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar from the appropriate substrates per minute under assay conditions. The enzymatic activities are expressed as units per gram of medium (U/ml) or (μmol/min). For the calculation of total enzyme activity, the first step is to calculate the molar absorption coefficient using the absorbance obtained. Using the molar absorption coefficient, the concentration of the substrate used can be calculated. Finally using following eq., enzyme activity can be calculated.

enzyme activity = 1 µmole of substrate released / 1 min of reaction

2.4.4 Enzyme production using seaweed cellulose

Three flasks containing 100 ml of production medium were inoculated by adding 2 ml of 24 hr old grown culture as an inoculum. The pH was adjusted to 7 and were studied for 24-72 hours. After every 24 hours 35 ml of the fermented broth was taken from one flask at a time, in a centrifuge tube and was centrifuged at 5000 rpm for 15 mins to extract the crude enzyme. Using crude enzyme total enzyme activity was calculated.

2.4.5 Protein determination

Protein concentrations in crude sample were determined by using a Lowry method (Lowry et al., 1951) with bovine serum albumin (BSA) as a standard.

2.4.6 Partial Purification of cellulase enzyme

1- Ammonium sulfate precipitation

Approximately 25 ml of the crude enzyme preparation was saturated to 80% with solid ammonium sulfate from SRL with 99.5% chemical purity. The mixture was then centrifuged, and the resulting pellet was dissolved in 10ml of PBS solution (Wingfield, 2001).

2- Dialysis

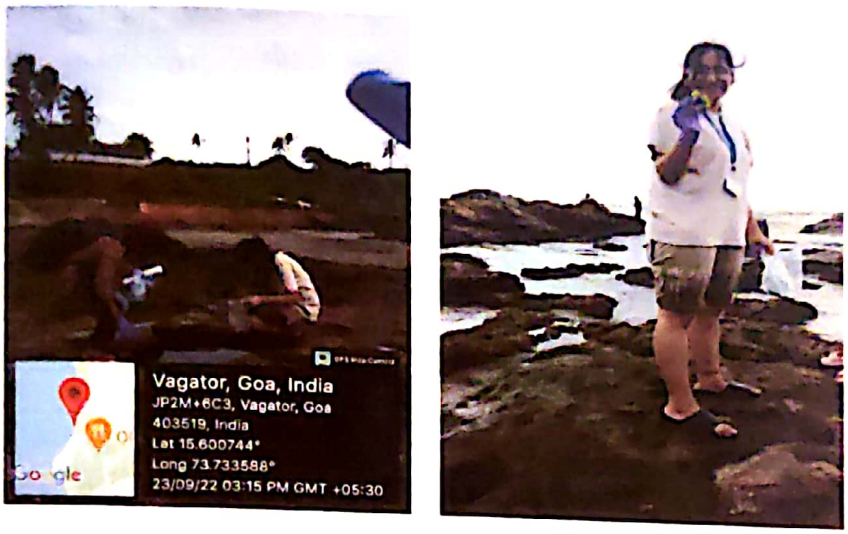
Eight millilitres of partly purified enzyme were dialysed against sodium phosphate buffer (pH-7.4). The protein fraction that was collected following the ammonium sulphate precipitation was dialysed using the pre-treated dialysis bag (pore size- 2.4 nm, dialysis membrane 50 by himedia) at 4˚C and thrice changes of buffer.

Chapter 3: Results and discussion-

3.1- Seaweed collection, identification, characterisation and extraction of cellulose

3.1.1 Seaweed collection

Approximately 650 g of fresh green seaweed was collected.



(a) (b)

Fig 1. (a) & (b) Collection of seaweed sample from the rocky shores of Vagator beach.

3.1.2 Seaweed characterisation

(i)- Morphological characterisation

The species was determined to be *Ulva* sp. based on morphological characteristics, including thallus colour, texture, nature of the margin, cell shape and size in surface.

Table-1 Morphological characteristics of collected seaweed

Characteristics	***Ulva* sp.**
Thallus colour and texture	Medium dark green, slimy thallus
Cell shape (surface view)	polygonal
Cell size (µm)	13-22
Dentate thallus margin	No

(ii) Elemental analysis (CHNS%)

CHNS% (carbon, hydrogen, nitrogen, sulphur) analysis of powdered seaweed was done using VarioMICRO cube. The results indicated that powdered seaweed fraction had following elemental composition: N [%] = 3.53%, C [%] = 32.88%, H [%] = 5.739% and S [%] = 3.377% (refer table 2).

Table 2- Table shows CHNS% present in seaweed.

S.no	**Weight (mg)**	**Name**	**Method**	**N [%]**	**C [%]**	**H [%]**	**S [%]**
1.	2.860	Cellulose	2mgChem80s NEV	3.53	32.88	5.739	3.377

3.2 Extraction of cellulose

From starting 650 gm of fresh *Ulva* biomass, 15g of dry cellulose was extracted (fig.2). The total dry weight of the *Ulva* biomass can be calculated as per literature (Carayo et al., 2008), which came to be 622.72g. Therefore, the total yield of dry cellulose was 2.40% (w/w) on dry weight basis.

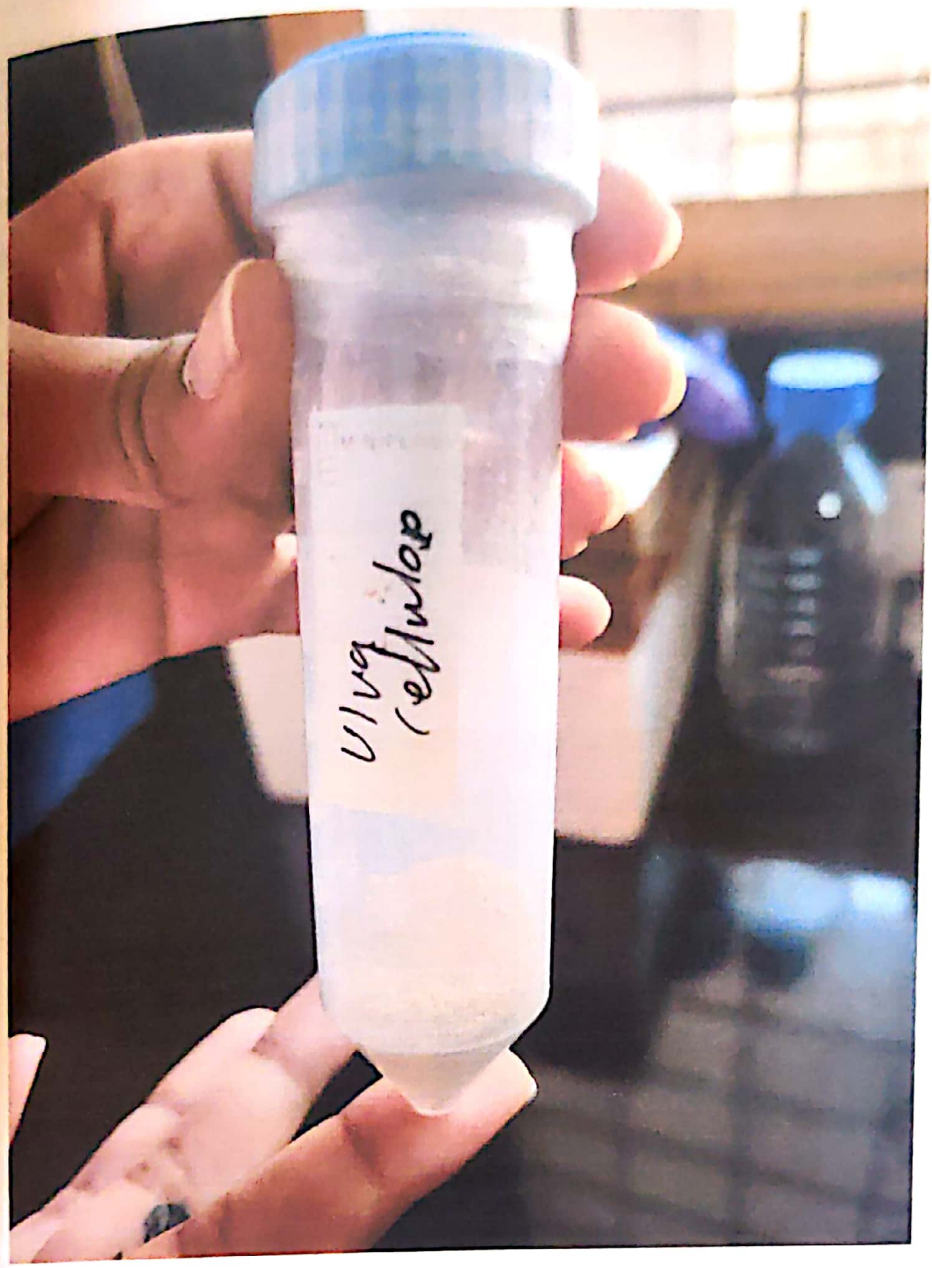


Fig.2- Extracted cellulose

3.2- Cellulose characterisation

3.2.1- Elemental analysis

The local growing circumstances have a significant impact on the chemical makeup of *Ulva*. Another factor that significantly affects the chemical makeup of algae is the age of the individual algae (Starko et al., 2019). The chemical composition of algae biomass will have impact on the composition of the cellulose extracted. CHNS% (carbon, hydrogen, nitrogen, sulphur) analysis of powdered cellulose was done using VarioMICRO cube. The results indicated that the obtained cellulose fraction had following elemental composition: N [%] = 2.72%, C [%] = 39.24%, H [%] = 5.662% and S [%] = 0.155% (refer table 3). A little amount of nitrogen and sulphur were present in the extracted cellulose could be due to presence of leftover fractions of protein and ulvan (from seaweed) in the cellulose.

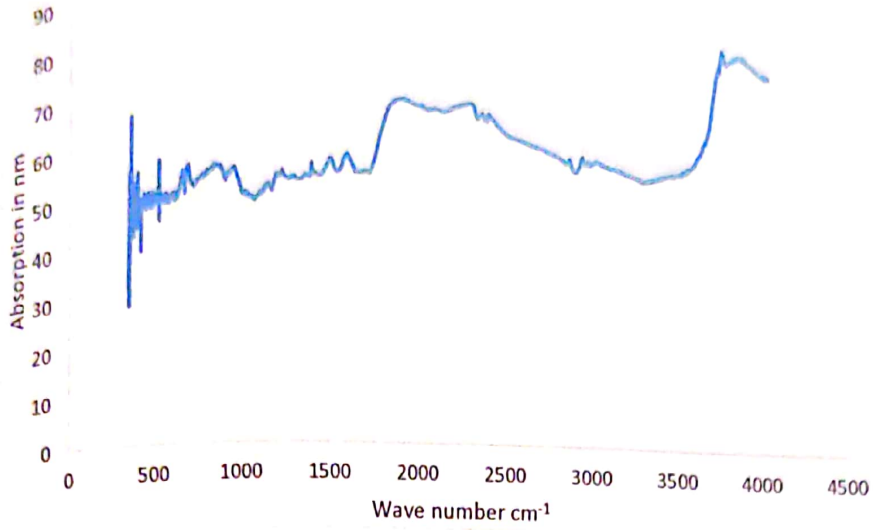
Table 3- Table shows CHNS% present in extracted cellulose.

S.no	Weight (mg)	Name	Method	N [%]	C [%]	H [%]	S [%]
1.	2.9100	Cellulose	2mgChem80s NEV	2.72	39.23	5.662	0.155

3.2.2- FTIR

Many of the distinctive peaks for polysaccharides may be seen in the FTIR spectra of the cellulose fractions (refer graph 1). The FTIR spectrum shows all the standard absorption typically seen in cellulose polymer. The broad bands at 3500-3200 cm-1 and the bands in the range 3000-2800 cm-1 correspond to the O-H stretching for the hydroxyl groups. The H-C-H scissor and tip vibrations are represented by the peaks at 1420 cm-1 and 1315 cm^{-1}, respectively. The symmetric stretching of C-O-C connections accounts for the abrupt peaks in the range of 1020–1050 cm-1 (*IR Spectrum Table*, 2023). The glycosidic bond, which is

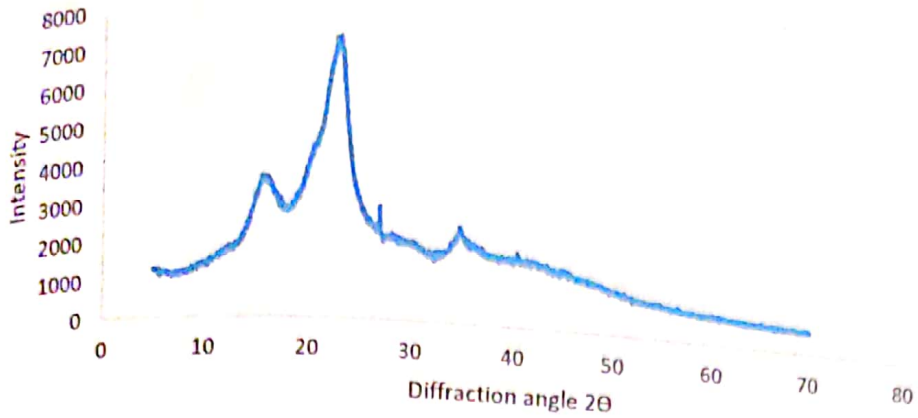
recognisable by the tiny peak at 895 cm-1, is a clear sign of the existence of polymeric polysaccharides like cellulose (Ververis et al., 2004).



Graph1: FTIR spectrum of cellulose fraction. Graph plotted for transmittance v/s wave number.

3.2.3- X-Ray Diffraction

The cellulose fractions crystal structure and crystallinity index were calculated using XRD (Park et al., 2010). In order to maintain the presence of crystalline regions, the extracted cellulose produced three peaks at 2 = 16°, 22°, and 34° (refer graph 2) that correspond to the crystal planes (110), (200), and (004), respectively (Sarko & Muggli, 1974) (French & Santiago Cintrón, 2013). The crystallinity index (CrI%) was calculated from the XRD diffractogram which came to be 56.7%. Hence was concluded that the extracted cellulose has crystalline structure.



Graph2: Graph plotted for intensity v/s diffracting angle showing X-ray diffraction of powdered cellulose fraction.

3.3- Isolation and screening for cellulase producing bacteria

A variety of microbial enzymes must take part in the complex of cellulosic material degradation. The ideal places to look for these bacteria are habitats that have these substrates (Huang & Monk, 2004). The spore formers were found to be more active markers of the enzyme when for testing for isolates with cellulolytic activity. Because soil is a rich source of diverse group of cellulolytic microbes due to the decomposition of leaflets, litter, and other plant wastes that contain significant levels of cellulosic matter, soil was chosen as a source for getting ideal cellulose generating organisms. Furthermore, the selection of this product is greatly influenced by its wide availability, simplicity of processing, and affordability. Four isolates were obtained from the soil sample which showed cellulase activity after being flooded by 1% congo red solution and maintained in pure culture in NA slants.

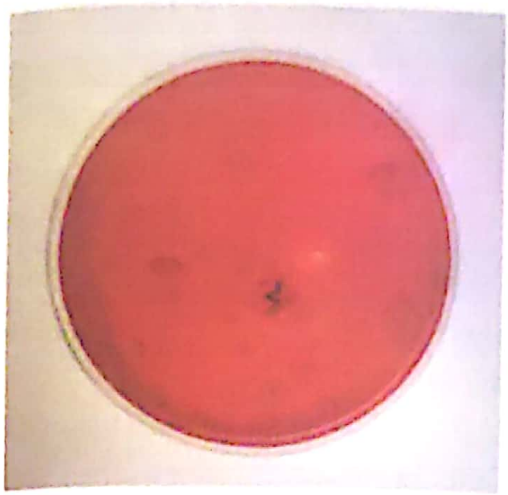


Fig 3. Bacterial colonies showing zone of clearance indicating cellulase activity after being flooded with 1% Congo red solution.

3.4- Identification of cellulase producing bacteria

3.4.1- Biochemical Characterisation

On CMC agar, the isolate formed white colonies. All isolates were identified as a Gram-positive bacterium out of those three were rod-shaped spores and one isolate was cocci and they produced cellulase enzyme. One isolate which showed maximum zone of clearance after plating on CMC and was further tentatively identified to be *Bacilli* species using biochemical tests.

Table 4- Various biochemical tests performed for gram positive bacterial isolate (ab1) which shows maximum cellulase activity

S.no	Biochemical test	Result
1	Endospore forming	+
2	Strict anaerobe	-
3	Catalase	+
4	Voges Praskauer	+
5	Methyl red	+
6	Motility	+
7	Citrate	+

3.4.2- Molecular characterisation

For identifying bacteria and species, the 16S-23S rRNA has been effective. Based on biochemical analysis and 16S rRNA sequencing, the selected cellulolytic bacteria in the current investigation have been tentatively identified. It is feasible to recognise and differentiate between bacterial species that are closely related. A single genomic band was seen in 1% agarose gel electrophoresis after genomic DNA from ab1 culture was extracted, as shown by gel documentation. On an agarose gel, a single discrete 1500 bp PCR amplicon band was visible. After running the nucleotide of the sequence obtained by 16S rRNA sequencing in BLAST, it was concluded the tentative identity of the culture is *Bacillus* sp. showing maximum percent identity of 92.06% with *Bacillus siamensis* strain DB071. It's evolutionary relatedness to other reported strains was clearly demonstrated by phylogenetic analyses that used the neighbours joining method.

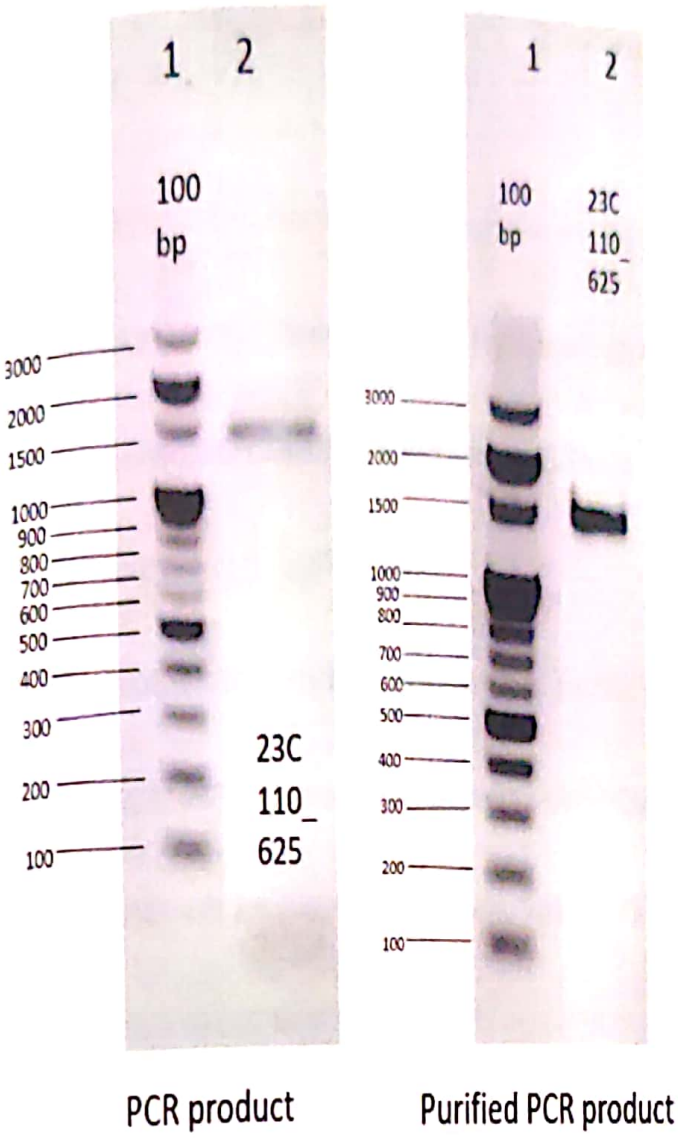


Fig. 4- Figure showing agarose gel electrophoresis result of PCR product of abl culture

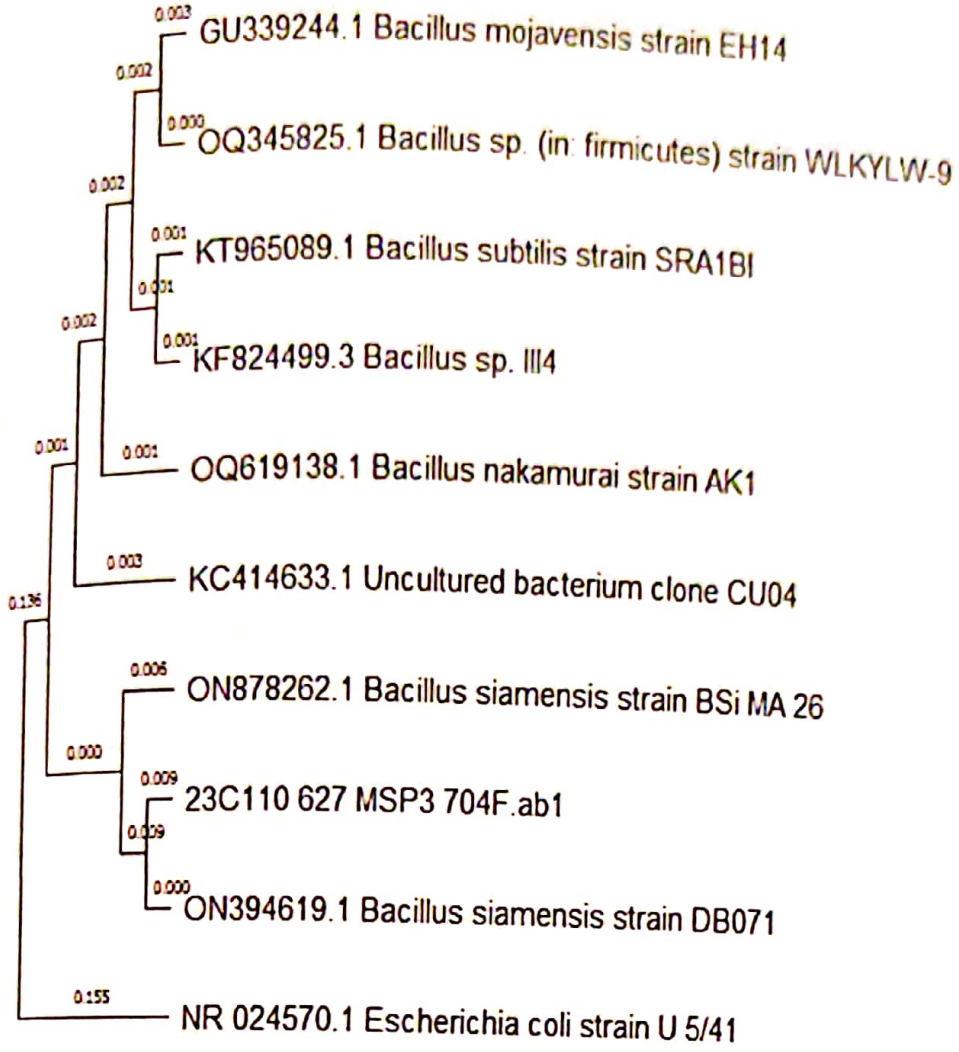


Fig. 5- Phylogenetic tree of the bacterial isolate, showing its relatedness with genus *Bacillus* sp. with *Escherichia coli* as an outgroup

3.5- Enzyme production

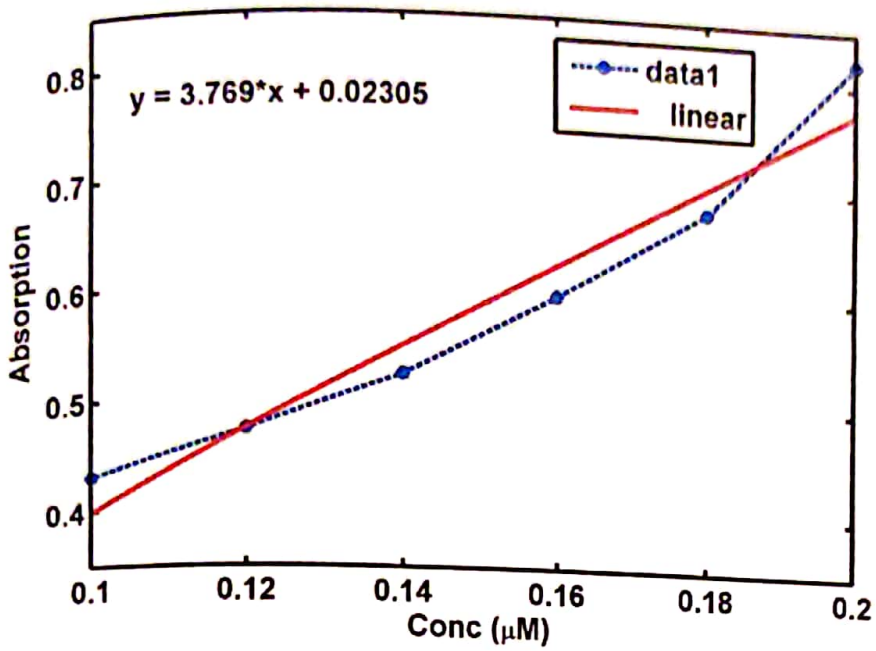
The optimum conditions for cellulase production by bacteria were given using extracted cellulose as substrate at pH 7 at room temperature 36°C.The culture that will be utilized to inoculate the fermentation medium must be in a sound, active state, be at its ideal size, maybe in the log phase, and convert substrate at a high rate. Since cellulase is an inducible enzyme, the type of production substrate has an impact on it. The design of the fermentation media included all the ideal characteristics, and cellulase production was completed.

Inoculating the isolated *Bacillus* species into the fermentation medium allowed for the measurement of cellulase production.

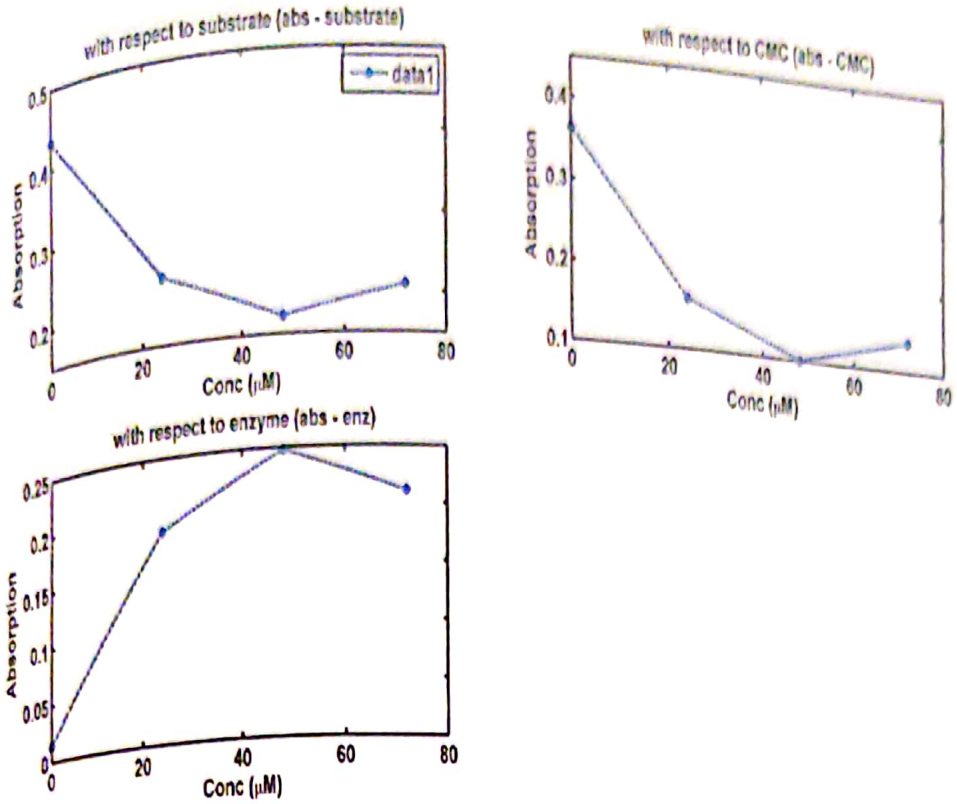
3.6- Enzyme activity assay

Enzyme activity of cellulase produced when extracted cellulose was given as substrate was recorded for 24-72 hours by extracting the crude enzyme from the fermentation broth. The crude enzyme solution was utilized for determination of enzyme activity. Cellulase activity was measured by the DNS method, from carboxy methyl cellulose (CMC), extracted cellulose (refer graph 4) and using glucose as standard (refer graph 3). The enzyme activity of the crude sample after 24 hours, 48 hours and 72 hours was found to be 0.01232µmole/min, 0.04125 µmole/min and 0.02µmole/min (refer graph 6). That shows on further incubation the values were found to decrease. Hence media optimisation may be carried out in future to optimise the cellulase production.

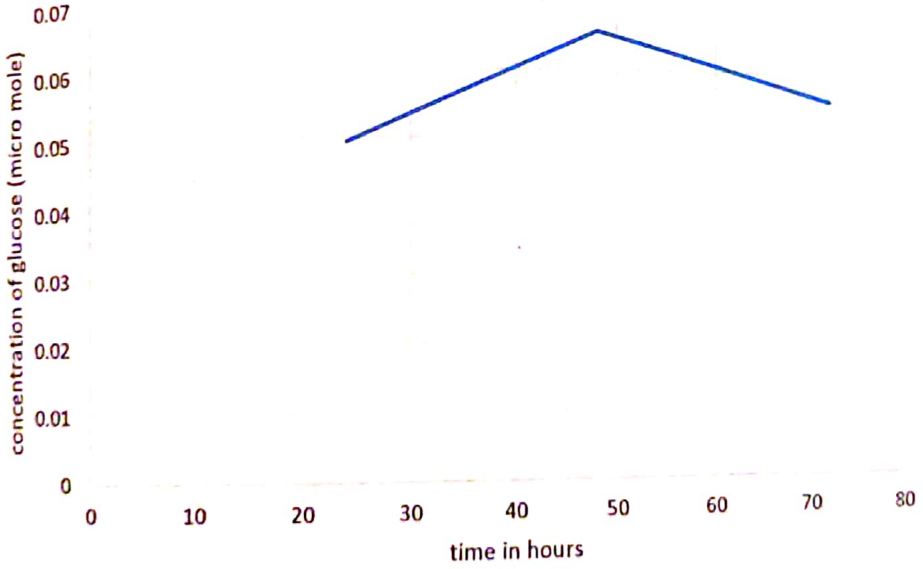
The protein concentrations in crude sample after 24 hours, 48 hours and 72 hours were determined with bovine serum albumin (BSA) as a standard which came to be 0.48mg/ml, 1mg/ml and 0.6mg/ml. It means after 48 hours of incubation the protein concentration was maximum and it was discovered that the enzyme activity had increased; however, with additional incubation, the values had decreased (Huang & Monk, 2004). With CMC as the substrate, the enzyme produced the maximum yield. This is thought to be because the isolated microbe can absorb it easily because it is less complicated.



Graph 3: Standard curve of glucose. Graph plotted for absorption v/s concentration of glucose



Graph 4: Graph showing comparison of absorption with control conditions with substrate control (extracted cellulose is used as substrate control with DNS reagent), CMC control and enzyme control (crude enzyme is used as enzyme control with DNS reagent)



Graph5: Graph showing concentration of glucose (samples taken after24,48 and 72 hours of incubation) with respect to time for the calculation of enzyme activity

3.7- Protein purification

Ammonium sulphate precipitation and dialysis were used to partially purify the extracellular cellulase generated by the isolated *Bacillus species* (Nakamura, 1982). After performing dialysis, the salts present in the partially purified cellulase were removed. Purified cellulase was obtained and was stored at -20°C.

Chapter 4: Conclusion

The goal of the current study was to extract cellulose from green seaweed using optimum conditions for the cellulolytic bacteria to produce cellulase.

The *Bacillus* species (ab1) that produced cellulase was isolated from a soil sample and tentatively identified using a variety of staining techniques, biochemical testing, and molecular technique. The maximum enzyme activity was shown after 48 hours of incubation. For every 1µmole of seaweed cellulose used, 0.04124 µmole cellulase was produced. The findings of my current work indicated that bacteria capable of producing cellulase can grow under standard conditions. The partial purification of the cellulase enzyme was carried out. The *Bacillus* species demonstrated the ability to transform cellulose into reducing sugars, which may be easily utilised in a variety of applications, including animal feed and as a feed stock for the synthesis of important organic chemicals. A prospective method for their large-scale manufacturing, as a potential food supplement, or in the pharmaceutical business is the utilisation of microbes for enzyme production. To conclude, the current research presents a comprehensive method for extraction cellulose from green seaweed and using it as a substrate for the production of cellulase by bacteria.

Bibliography

Bhat, M. K. (2000). Cellulases and related enzymes in biotechnology. *Biotechnology Advances*, *18*(5), 355–383. https://doi.org/10.1016/S0734-9750(00)00041-0

Camassola, M., & Dillon, A. J. P. (2007). Production of cellulases and hemicellulases by Penicillium echinulatum grown on pretreated sugar cane bagasse and wheat bran in solid-state fermentation. *Journal of Applied Microbiology*, *103*(6), 2196–2204. https://doi.org/10.1111/j.1365-2672.2007.03458.x

Carayo, C., Culango, K., Estrera, D., Largo, D., & Dy, D. (2008). Correlation of inorganic nutrients to the standing crop of "green tide" algae during the peak growth season in eastern Mactan island, central Philippines. *The Philippine Scientist*, *42*. https://doi.org/10.3860/psci.v42i0.357

Dominguez, H., & Loret, E. P. (2019). Ulva lactuca, A Source of Troubles and Potential Riches. *Marine Drugs*, *17*(6), 357. https://doi.org/10.3390/md17060357

Ferreira, R. da G., Azzoni, A. R., & Freitas, S. (2018). Techno-economic analysis of the industrial production of a low-cost enzyme using E. coli: The case of recombinant β-glucosidase. *Biotechnology for Biofuels*, *11*(1), 81. https://doi.org/10.1186/s13068-018-1077-0

French, A. D., & Santiago Cintrón, M. (2013). Cellulose polymorphy, crystallite size, and the Segal Crystallinity Index. *Cellulose*, *20*(1), 583–588. https://doi.org/10.1007/s10570-012-9833-y

Huang, X. P., & Monk, C. (2004). Purification and characterization of a cellulase (CMCase) from a newly isolated thermophilic aerobic bacterium Caldibacillus cellulovorans gen. Nov., sp. Nov. *World Journal of Microbiology and Biotechnology*, *20*(1), 85–92. https://doi.org/10.1023/B:WIBI.0000013316.12730.e7

Immanuel, G. (2006, April 18). *Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment | SpringerLink*. https://link.springer.com/article/10.1007/BF03325904

IR Spectrum Table. (n.d.). Retrieved April 24, 2023, from https://www.sigmaaldrich.com/IN/en/technical-documents/technical-article/analytical-chemistry/photometry-and-reflectometry/ir-spectrum-table

Koomnok, C. (2006). *Koomnok: Selection of cellulase producing thermophilic fungi—Google Scholar*. https://scholar.google.com/scholar_lookup?title=Selection%20of%20cellulase%20producing%20thermophilic%20fungi&author=C.%20Koomnok

Lee, Y.-J., Kim, B.-K., Lee, B.-H., Jo, K.-I., Lee, N.-K., Chung, C.-H., Lee, Y.-C., & Lee, J.-W. (2008). Purification and characterization of cellulase produced by Bacillus amyoliquefaciens DL-3 utilizing rice hull. *Bioresource Technology*, *99*(2), 378–386. https://doi.org/10.1016/j.biortech.2006.12.013

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, *193*(1), 265–275.

Lü, Y., Wang, X., Li, N., Wang, X., Ishii, M., Igarashi, Y., & Cui, Z. (2011). Characterization of the effective cellulose degrading strain CTL-6. *Journal of Environmental Sciences*, *23*(4), 649–655. https://doi.org/10.1016/S1001-0742(10)60460-3

Maki, M., Leung, K. T., & Qin, W. (2009). The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *International Journal of Biological Sciences*, *5*(5), 500–516.

Nakamura, K. (1982). Isolation and identification of Crystalline Cellulose Hydrolyzing Bacterium and its Enzymatic Properties: *Journal of Fermentation Technology*, *60*(4), 343–348.

Park, S., Baker, J. O., Himmel, M. E., Parilla, P. A., & Johnson, D. K. (2010). Cellulose crystallinity index: Measurement techniques and their impact on interpreting cellulase performance. *Biotechnology for Biofuels*, *3*(1), 10. https://doi.org/10.1186/1754-6834-3-10

Patagundi, B. I., Shivasharan, C. T., & Kaliwal, B. B. (2014). *Original Research Article Isolation and Characterization of Cellulase producing bacteria from Soil*.

segal, l. (1959, October). *An Empirical Method for Estimating the Degree of Crystallinity of Native Cellulose Using the X-Ray Diffractometer—L. Segal, J.J. Creely, A.E. Martin, C.M. Conrad, 1959*. https://journals.sagepub.com/doi/10.1177/004051755902901003

Siddhanta, A. K., Kumar, S., Mehta, G. K., Chhatbar, M. U., Oza, M. D., Sanandiya, N. D., Chejara, D. R., Godiya, C. B., & Kondaveeti, S. (2013). Cellulose Contents of Some Abundant Indian Seaweed Species. *Natural Product Communications*, *8*(4), 1934578X1300800423. https://doi.org/10.1177/1934578X1300800423

Starko, S., Bailey, L. A., Creviston, E., James, K. A., Warren, A., Brophy, M. K., Danasel, A., Fass, M. P., Townsend, J. A., & Neufeld, C. J. (2019). Environmental heterogeneity mediates scale-dependent declines in kelp diversity on intertidal rocky shores. *PLoS ONE*, *14*(3), e0213191. https://doi.org/10.1371/journal.pone.0213191

Wahlström, N., Edlund, U., Pavia, H., Toth, G., Jaworski, A., Pell, A. J., Choong, F. X., Shirani, H., Nilsson, K. P. R., & Richter-Dahlfors, A. (2020). Cellulose from the green macroalgae Ulva lactuca: Isolation, characterization, optotracing, and production of cellulose nanofibrils. *Cellulose*, *27*(7), 3707–3725. https://doi.org/10.1007/s10570-020-03029-5

Wingfield, P. T. (2001). Protein Precipitation Using Ammonium Sulfate. *Current Protocols in Protein Science / Editorial Board, John E. Coligan ... [et Al.]*, *APPENDIX 3*, Appendix-3F. https://doi.org/10.1002/0471140864.psa03fs13

Appendix

1- CMC Agar media composition per 1000 ml

Part-1

$NH_4H_2PO_4$ - 1g

KCl – 0.2g

$MgSO_4.7H_2O$ - 1g

Yeast extract – 1g

Part – 2

CMC (Carboxy Methyl Cellulose) – 5g

Agar powder – 20g

2- Nutrient agar media composition per 1000 ml

NaCl – 5g

Peptone – 5g

Yeast extract – 3g

Agar powder – 20g

3- Production media composition per 1000 ml

$NH_4H_2PO_4$ – 1g

KCl - 0.2g

$MgSO_4.7H_2O$ – 1g

yeast extract – 1g

cellulose – 5g

4- **50Mm Sodium Phosphate Buffer composition per 1000 ml**

$Na_2HPO_4.7H_2O$ – 7.7g

$NaH_2PO_4.H_2O$ – 2.9g