International Journal of Engineering Science, Advanced Computing and Bio-Technology
Vol. 6, No. 3, July – September 2015, pp. 74 - 96

Optimization of Production Parameters for the Synthesis of Lignocellulolytic Enzymes from Marine Actinomycetes

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Abstract: *Among the marine organisms, actinomycetes are the group of bacteria that are widely distributed. They are known to play a very supporting role in the degradation of organic matter in the production of lignocellulolytic enzymes. In the present study eight actinomycetes strains (SV 7701, SV 7702, SV 7703, SV 7704, SV 7705, SV 7706, SV 7707 and SV 7708) were screened for their potential to produce lignocellulolytic enzymes (cellulase, laccase and peroxidase). The strains were tested for enzyme production by qualitative plate assays. The strain SV7702 was selected as the maximum producer and selected for optimization, production and characterization of cellulase enzyme. The production parameters such as pH, temperature, carbon source, nitrogen source, incubation period and substrate concentration were optimized to be 5, 45°C, xylose, urea, 6 and 0.8%, respectively. The enzyme thus produced under optimized conditions was characterized by SDS-PAGE and Native-PAGE. Thus, the current study produced industrially important cellulose using the selected actinomycetes strain.*

Keywords: *Actinomycetes, Lignocellulolytic enzymes, Optimization, SDS-PAGE*

1. Introduction

1.1 Actinomycetes

Actinomycetes are prokaryotic, spore forming, gram positive bacteria. Their filamentous nature, branching pattern and conidia formation are similar to fungi, for this reason, they are known as “ray fungi”. The spore size, spore characters, prokaryotic nuclei and susceptibility to antibacterial antibiotics are similar to bacterial characteristics. For this reason, they are known as filamentous bacteria (Balakrishnan et al., 2012). They belong to the order Actinomycetales (Superkingdom: Bacteria, Phylum: Firmicutes, Class: Actinobacteria, Subclass: Actinobacteridae). They have high G+C (>55%) content in their

Received: 21 May, 2015; Revised: 18 August, 2015; Accepted: 15 September, 2015

DNA (Gurung et al., 2009). Actinomycetes are best known for their ability to produce antibiotics, which comprise a group of branching unicellular microorganism. The colonies have pastel colors, soil-like odour, hard and stick into the agar (Kalyani et al., 2012).

1.2 Sources of Actinomycetes

Actinomycetes form a large and important segment of the microflora of most natural environments. Soils, freshwater, lake and river bottoms, manures and compost contain an abundance of these organisms. They are of universal occurrence in nature, living and multiplying in both cold and tropical zones and have been reported to occur even under the most extreme conditions of the desert. The temperate zones are, however, generally most favourable for their development (Strzelczyket al., 1967). Actinomycetes are the most widely distributed group of microorganisms in nature which primarily inhabit the soil (Kumar et al., 2010).The marine environment is still an untapped source of the diverse group of actinomycetes with unique biological functions (Mohanrajet al., 2013). Among the microorganisms, marine actinomycetes have been an excellent source of new compounds and their isolation all around the globe, from shallow coastal to the deepest sediments. Marine environment is an enormous treasure trove of marine actinomycetes resources (Selvamet al., 2011). A little is known about the actinomycetes diversity of marine sediments, which is an inexhaustible resource that has not been adequately exploited (Remya et al., 2008).

1.3 Compounds Produced by Actinomycetes

Actinomycetes have been exploited successfully for their biologically potential secondary metabolites. They provide diverse group of antimicrobial metabolites notably glycopeptides, beta-lactams, aminoglycosides, polyenes, polyketides, macrolides, actinomycins and tetracyclines. Marine actinomycetes are a potential source of novel compounds as the environmental conditions of the sea are entirely different from that of terrestrial conditions (Mohanrajet al., 2013). They have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds. These searches have been remarkably successful and approximately two-thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes. Almost 80% of the world's antibiotics are known to come from Actinomycetes, mostly from the genera Streptomyces and Micromonospora. Presently, most of the disease-causing bacteria has become resistant to

most of the antibiotics. Staphylococcus aureus is commonly known pathogen that has developed resistance to most classes of antibiotics (Pandey et al., 2011). Marine microorganisms are increasingly becoming an important source in the production of medical and industrially important enzymes. It could provide rare and unique microbial products, particularly enzymes that could be safely used for human, therapeutic purposes (Selvamet al., 2011).

1.4 Lignocellulose

Lignocellulose is the primary structural component of woody plants and non-woody plants such as grass that represents a significant source of renewable organic matter. Lignocellulose consists of lignin, hemicellulose and cellulose. The chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value. Large amounts of lignocellulosic wastes are generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro-industries, and they pose an environmental pollution problem. Sadly, much of the lignocellulose waste is often disposed of by biomass burning, which is not restricted to developing countries alone, but is considered a global phenomenon. However, the massive amounts of residual plant biomass considered as "waste" can potentially be converted into various different value-added products including biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds and human nutrients (Howard et al., 2003). Since the enzymatic hydrolysis of woody lignocelluloses is gaining increased research attention due to its immense potential for transformation into fermentable sugars, the supply of highly active, inexpensive cellulolytic enzymes is indispensable in developing an economically feasible biotechnical process (Shimokawaet al., 2012).

1.5 Lignocellulolytic Enzymes

Cellulose, the most abundant constituent of the plant cell wall, is a linear polysaccharide composed of ß-1-4-linked glucose molecules. Efficient hydrolysis of cellulose requires the action of a compound of cellulolytic enzymes that work synergistically: exo-1-4- β-D-glucanases, endo-1-4- β-D-glucanases and 1-4- β-D-glucanases. At present, there is an increasing demand for these enzymes, especially for bioconversion of agricultural resources and ethanol production. Therefore, development of more efficient enzyme preparations at low cost is required. Production of highly active and stable cellulolytic complexes depend on the microorganism and the procedure used to

obtain the protein, especially in relation to the composition of media for cultivation; this can affect the growth and product yield due to substances that can act as inducers, activators, inhibitors and repressors (Sette et al., 2008). Lignin represents about 15-25% of lignocellulosic biomass and is a large cross-linked macromolecule composed of three monomers: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. The enzymes responsible for lignin degradation are mainly lignin-peroxidase (LiP), manganese peroxidase (MnP), laccases and hydrogen peroxide producing enzymes (Sette et al., 2008). Although a broad range of enzymes has been reported to be involved in the tedious process of lignin degradation only a few enzymes like Lignin peroxidase, manganese peroxidase and laccases play significant role and each of these enzymes exhibit particular mode of action (Niladevi, 2008).

1.6 Applications of Lignocellulolytic Enzymes

The demand for industrial microbial enzymes is ever increasing due to their use in a wide variety of processes. Lignocellulolytic enzymes have potential applications in a significant number of fields, including the chemical, fuel, food, agricultural, paper, laundry, animal feed, textile and cosmetic, industrial sectors. One of the most significant potential applications of lignocellulolytic enzymes is fuel production from agricultural and forest wastes as an alternative renewable energy resource (Sette et al., 2008; Elisashviliet al., 2006).Although lignolytic enzymes play a crucial role in the global carbon cycle, one of the most important current applications of these enzymes is related to environmental remediation, a process in which biological systems are used to degrade or neutralize pollutants or to decolourise dyes (Sette et al., 2008)

2. Materials and Methods

2.1 Maintenance of Actinomycetes Culture

Eight actinomycetes cultures were obtained from ARMATS BIOTEK, Chennai namely SV 7701, SV 7702, SV 7703, SV 7704, SV 7705, SV 7706, SV 7707, SV 7708. They were sub-cultured on Starch Casein Agar (SCA) plates and incubated for 7days at room temperature. The strains were pure cultured on SCA slants.

2.2 Screening of Actinomycetes for Enzyme Activity

2.2.1 Qualitative Screening

Cellulase

The isolates were streaked (single streaking) on SCA plates amended with CMcellulose (1%) and incubated for 24-48hrs at room temperature. The plates were flooded with 0.3% Congo red solution for 10mins. Then it is washed with water and flooded with 1N NaCl as a destaining solution. Cellulase production is visualized by a translucent zone around the colonies.

Laccase

The isolates were streaked (single streaking) on SCA plates amended with Tween 80 (1%) and incubated for 24-48hrs at room temperature. The plates were incubated at 25°C in dark place for five days. The appearance of intense brown colour around the colonies confirmed the laccase production.

Peroxidase

The isolates were streaked (single streaking) on SCA plates amended with H2O2 (1%) and incubated for 24-48hrs at room temperature. The plates were flooded with an electron donor dye (O-dianisidine) for 10mins. The formation of pink colour around the colonies confirmed the peroxidase reaction.

2.3 Quantitative Screening

The isolate showing maximum enzyme production was selected and was subjected to quantitative estimation of the specific enzyme.

Quantitative estimation of cellulose activity - DNS Method

The assay mixture consisting of 0.1 ml culture filtrate and 0.9 ml of suspension of 1% carboxy methyl cellulose (CMC) prepared in 100 mM sodium citrate buffer (pH - 5.0) was prepared and incubated at 55°C in a water bath with constant shaking.Controls, without enzyme, substrate and with boiled enzyme were maintained.After 15mins, the reducing sugar formed in the reaction mixture was estimated by the method of Miller (1959) using dinitro salicylic acid (DNS) reagent. The reaction was terminated by the

addition of 1ml DNS to each tube.The tubes were boiled in a water bath for 10mins after which 1ml of 40% sodium potassium tartrate solution and 2ml of water were added to all the tubes.The liberated glucose is measured by spectrophotometer at 540nm.The enzyme activity was determined using glucose as standard.

Enzyme Activity

One unit of enzyme activity is defined as the amount of enzyme that released 1µmol of glucose/min/ml of reaction mixture under standard assay condition. Specific cellulase activity is expressed as units per milligram protein.

$$\text{Enzyme activity} = \frac{\text{Amount of sugar released}}{\text{ml of enzyme} \times \text{min}}$$

2.4 Characterization of the best-screened isolate

The potent strain selected from primary screening was characterized by morphological, biochemical methods.

Morphological Characterization

The morphological method consists of macroscopic and microscopic characterization.

Macroscopical Characterization

Macroscopically the Actinomycetes isolates were differentiated by their colony characters, e.g. size, shape, color, consistency, etc.

Microscopical Characterization

For the microscopy, the isolates were grown by cover slip culture method and differentiated by Gram staining.

Biochemical Characterization

The bacterial isolates were characterized biochemically by Oxidase test, Catalase test, Gelatin liquefaction, Starch hydrolysis, Cellulose degradation, Milk coagulation and Sodium chloride tolerance.

Oxidase Test

Oxidase test was performed by rubbing the actinomycetes strain (SV 7702) using sterile toothpick on the oxidase disc that contains the oxidase reagent. The disc was observed after 10-30secs for dark purple colour around the edge of the organism. Dark purple colour indicates active, and no colour changes indicate negative.

Catalase Test

A loop full of actinomycetes strain from SCA slant was stirred on 30.0 v/v hydrogen peroxide and observed for evolution of gas. Formation of gas bubble is positive, and no formation of gas bubbles is negative.

Cellulose Degradation

1% of Carboxy Methyl Cellulose (CMC) was added to the SCA media. The plates were inoculated and incubated for seven days. Cellulose degradation was visually observed using Congo red dye.

Starch Hydrolysis

This test was done to observe the activity of amylase enzyme. It is used to determine the ability of an organism to hydrolyse (breakdown) starch. Single streak of the organism was made on a starch agar plate. It is incubated at optimal temperature for 24-48hours.A small amount of Gram's Iodine was poured onto the plate, and the plate was gently rotated. Iodine is an indicator of starch. In the presence of starch-iodine will turn blue or black. Positive indicates a zone of clearing appears adjacent to the streak line and negative indicates no clearing; only a blue or black area surrounding the colony.

Gelatin Liquefaction

This test was done to study the activity of enzyme gelatinase. Gelatin is a protein produced by hydrolysis of collagen, a major component of connective tissue and tendons

in humans and other animals. Below temperature of 25°C, gelatin will maintain its gel properties and exist as a solid at temperatures above 25°C, gelatin is liquid. Liquefaction is accomplished by some microorganisms capable of producing gelatinase, which acts to hydrolyze this protein to amino acids. Once this degradation occurs, even very low temperatures of 4°C will not restore the gel characteristic.SC broth consists of nutrient supplemented with 12% gelatin results in a stiff medium and also serves as the substrate for the activity of gelatinase. Gelatin liquefaction was studied by inoculating the strain on gelatin tubes and incubated at 37°C for seven days. The extent of liquefaction was recorded after keeping the tubes in cold conditions (5-10°C) for an hour. Cultures that remain liquefied were indicative of gelatin hydrolysis.

Milk Coagulation

This test was done to study the activity of caseinase enzyme. The skimmed milk tubes were inoculated and incubated at 37°C for seven days. The extent of coagulation was visually observed on the agar slants.

Sodium Chloride Tolerance

This test was very important for to understand the native nature of the marine actinomycetes isolates. Different concentrations of sodium chloride (5, 10, 15, and 20%) solution were added to the starch casein agar (SCA) medium to check the sodium chloride tolerance test. The isolate was streaked on the agar medium, incubated at 37°C for seven days, and the presence or absence of growth was observed.

2.5 Optimization

The effects of Carbon, Nitrogen, Incubation period, Substrate concentration, Temperature and pH on the production of the enzyme were studied. These were carried out by cultivating the isolate at different days (2,4,6,8) different temperatures and different pH values (5-8). The cellulase activity and the protein content were assayed.

2.5.1 Effect of Carbon Source

Experiments were carried out to investigate the effect of different carbon sources such as fructose, starch, sucrose and xylose on the production of cellulase enzyme. The enzyme activity was determined by DNS method.

2.5.2 Effect of Nitrogen Source

Experiments were carried out to investigate the effect of different nitrogen sources such as casein, peptone, urea and yeast extract on the production of cellulase enzyme. The enzyme activity was determined by DNS method.

2.5.3 Effect of Incubation Period

An experiment was conducted to optimize the days of incubation of the culture medium by measuring absorbance (540nm) on different days of incubation (2,4,6,8). The enzyme activity was determined by DNS method.

2.5.4 Effect of Substrate Concentration

An experiment was conducted to optimize the substrate concentration of the fermentation medium by measuring the absorbance (540nm) at different concentrations of CMC (0.2, 0.4, 0.6, 0.8 and 1%). The enzyme activity was determined by DNS method.

2.5.5 Effect of pH

An experiment was conducted to determine the most suitable pH of the fermentation medium by adjusting the pH of the culture medium at different levels in the range of pH 5 to 8 using different buffers. The enzyme activity was determined by DNS method.

2.5.6 Effect of Temperature

An experiment was conducted to determine the most suitable temperature of the fermentation medium by placing the culture medium at different temperature levels such as refrigerator (8^{o}C), room temperature (27^{o}C), incubator (45^{o}C) and hot air oven (65^{o}C). The enzyme activity was determined by DNS method.

2.6 Purification of Enzyme

2.6.1 Ammonium Sulphate Precipitation

Partial purification of cellulase enzyme was achieved by ammonium sulphate precipitation followed by dialysis. 100 ml of cell-free extract was saturated with ammonium sulphate up to 80%. The content was incubated overnight and centrifuged at 5000 rpm for 20 min. Supernatant was collected and saturated up to 90% with ammonium

sulphate. Then the content was centrifuged at 5000 rpm for 20mins and pellet was collected for further analysis.

2.6.2 Dialysis

The enzyme mixture was transferred to a dialysis bag and immersed in phosphate buffer at 4°C for 24hrs. Buffer was continuously stirred using a magnetic stirrer throughout the process. Buffer was changed three times during the process in order to obtain proper purification.

2.7 Characterization of Enzyme

2.7.1 Electrophoretic studies

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-Polyacrylamide gel electrophoresis was performed on slab gel with separating and stacking gels (10 & 5 % w/v) by the method of (Laemmli, 1970).

Staining of separated proteins

At the end of electrophoresis, gel was removed and stained with silver staining method. After staining, the gels were stored in 7 % (v/v) acetic acid.

Native Polyacrylamide Gel Electrophoresis:

Polyacrylamide gel electrophoresis of the purified laccase was carried out to analyze the protein profile of the enzyme by using Bio-Rad Electrophoresis Apparatus, USA. The following stock solutions were prepared, filtered, stored in dark color bottles and refrigerated.

3. Results and Discussion

3.1 Subculturing of Actinomycetes

The actinomycete isolates were subcultured on SCA plates and were cultured stored on Starch Casein Agar (SCA) slants at 8°C.

1 – SV7701 2 – SV7702 3 – SV7703 4 – SV7704

5 – SV7705 6 – SV7706 7 – SV7707 8 – SV7708

Figure 1: Actinomycetes strain

3.2 Screening of Actinomycetes for enzyme production

3.2.1 Qualitative Screening

3.2.1.1 Cellulase

The streaked SCA plates amended with 0.5% Carboxy Methyl Cellulose (CMC) was stained assayed with Congo red dye. Zone of clearance was measured for all the strains in which SV 7702 shows maximum (1.7cm).

Strain Number	Zone of clearance (cm)
SV 7701	0.6
SV 7702	1.7
SV 7703	0.8
SV 7704	0.6
SV 7705	1.2
SV 7706	1.4
SV 7707	0.3
SV 7708	0.1

Strain Number Zone of clearance (cm)

Table 1: Zone of clearance for Cellulase

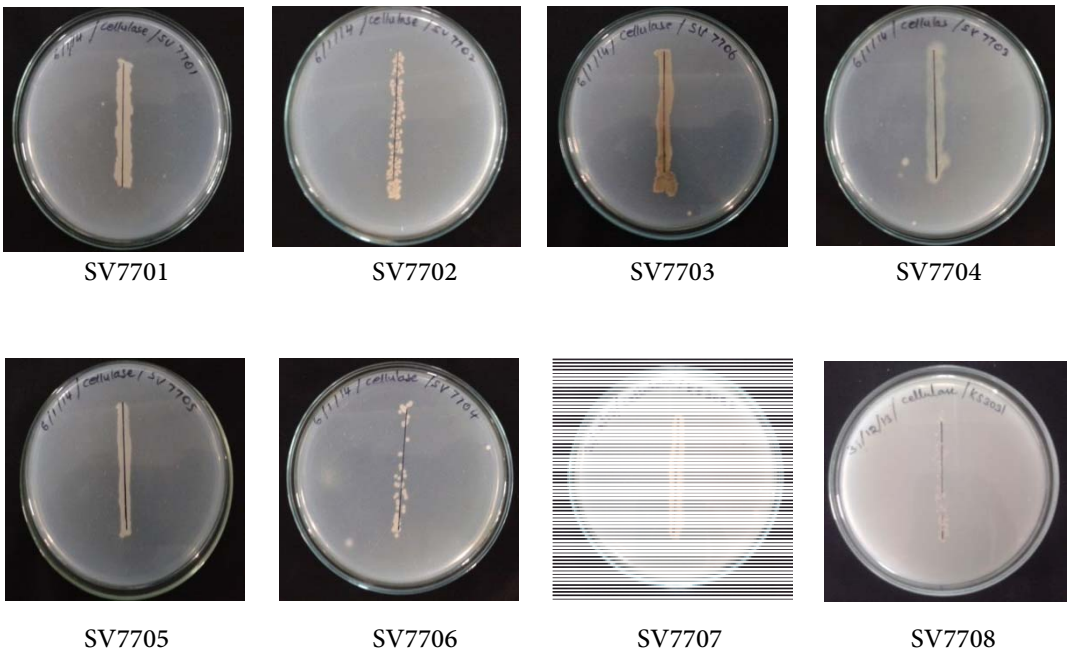


Figure 2: Cellulase before staining

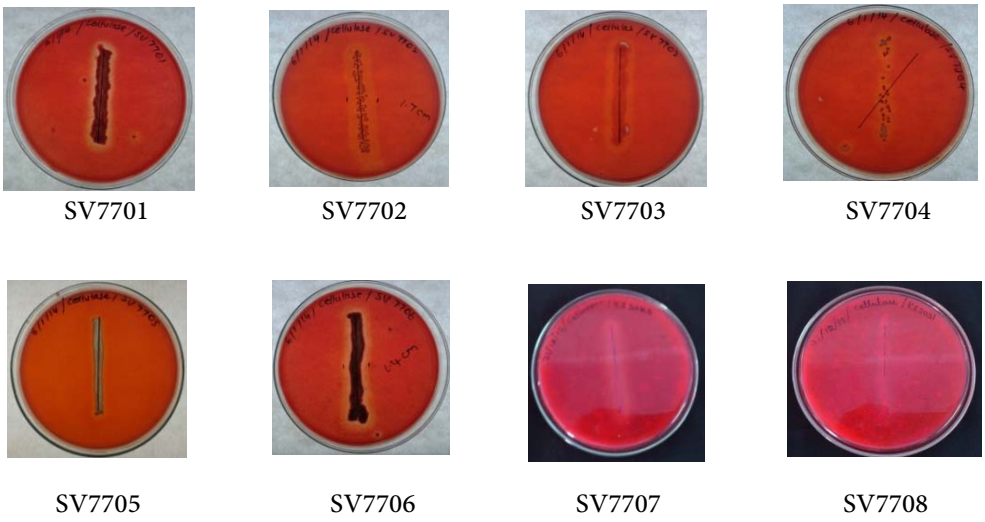


Figure 1: Cellulase-After Staining

3.2.1.2 Laccase

Laccase was screened by streaking the actinomycetes strains on SCA plates amended with suitable substrate 10mM Guaiacol. After five days of incubation, there are no brown color colonies observed. Results showed that all the eight strains were laccase negative.

SV7701 SV7702 SV7703 SV7704

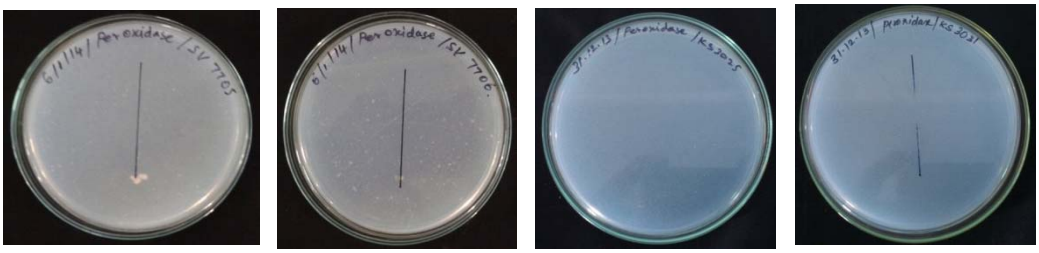
SV7705 SV7706 SV7707 SV7708

Figure 2: Laccase Activity

3.2.1.3 Peroxidase

Peroxidase was screened by streaking the actinomycetes strains on SCA plates amended with suitable substrate 10mM Hydrogen Peroxide (H2O2). After 5days of incubation, the plates were assayed with an electron donor dye. The results showed that peroxidase activity was negative.

SV7701 SV7702 SV7703 SV7704



SV7705 SV7706 SV7707 SV7708

Figure 3: Peroxidase Activity

3.3 Characterization

3.3.1 Morphological Characterization

3.3.1.1 Macroscopical Characterization

Macroscopically the Actinomycetes isolates were differentiated by their colony characters. They were tabulated below.

Strain Number	Growth	Aerial Mycelium	Substrate Mycelium
SV 7701	Rapid	Whitish Pink	Light Yellow
SV 7702	Moderate	Peachish White	Peachish Yellow
SV 7703	Poor	Pale Yellow	Sandal
SV 7704	Moderate	Greyish white	Medium Sandal
SV 7705	Rapid	Grey colour	Light Sandal
SV 7706	Rapid	White colour	Light Brown
SV 7707	Moderate	Grey	Brownish Grey
SV 7708	Moderate	Greyish White	Brown

Table 2: Macroscopical Characterization of actinomycetes

3.3.1.2 Microscopical Characterization

3.3.1.2.1 Cover Slip Method

After 7days of incubation, the cover slip was visualized under light microscope (40X). The spore chain morphology of SV 7702 was given below.

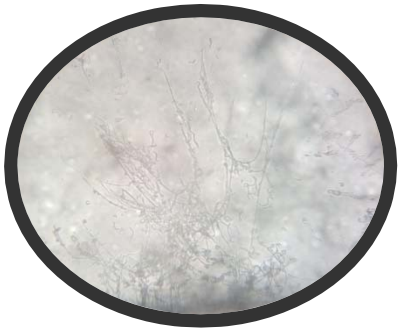


Figure 6: Microscopical Observation of SV 7702

3.3.1.2.2 Gram Staining

The result of the gram staining indicated that the strain SV 7702 was gram positive.

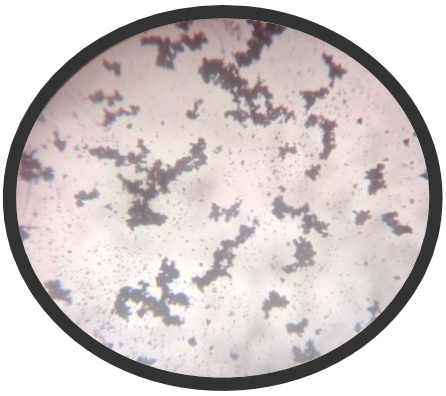


Figure 7: Gram Staining

3.3.2 Biochemical Characterization

Biochemical tests such as oxidase test, catalase test, cellulose degradation, starch hydrolysis, coagulation of milk, gelatin liquefaction and sodium chloride tolerance were

conducted for the potential isolate SV 7702. Results of the biochemical tests were tabulated.

Biochemical Test	Result
Oxidase Test	-Ve
Catalase Test	+Ve
Cellulose Degradation	+Ve
Starch hydrolysis	+Ve
Gelatin Liquefaction	-Ve
Milk Coagulation	-Ve

Table 3: Biochemical Characterization Results

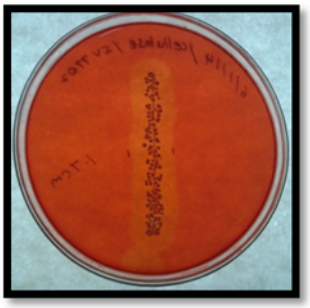


Figure 8: Cellulase Degradation

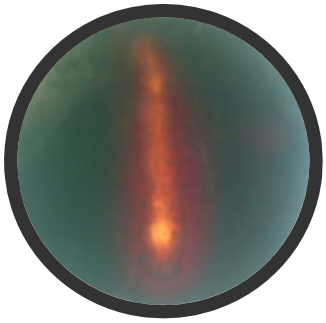


Figure 9: Starch Hydrolysis

3.4 Optimization

Media optimization was done to increase the yield of a fermentation process. The effects of Carbon, Nitrogen, Incubation period, Substrate concentration, Temperature and pH on the production of the enzyme were studied.

3.4.1 Effect of Incubation Period

The incubation period was optimized by varying the days of incubation (2, 4, 6 and 8). Results of the process showed that six days of incubation period provides maximum enzyme activity.

Days Of Incubation	Enzyme activity (U/ml)
2	0.027
4	0.0727
6	0.097
8	0.089

Table 4: Days of Incubation Observation

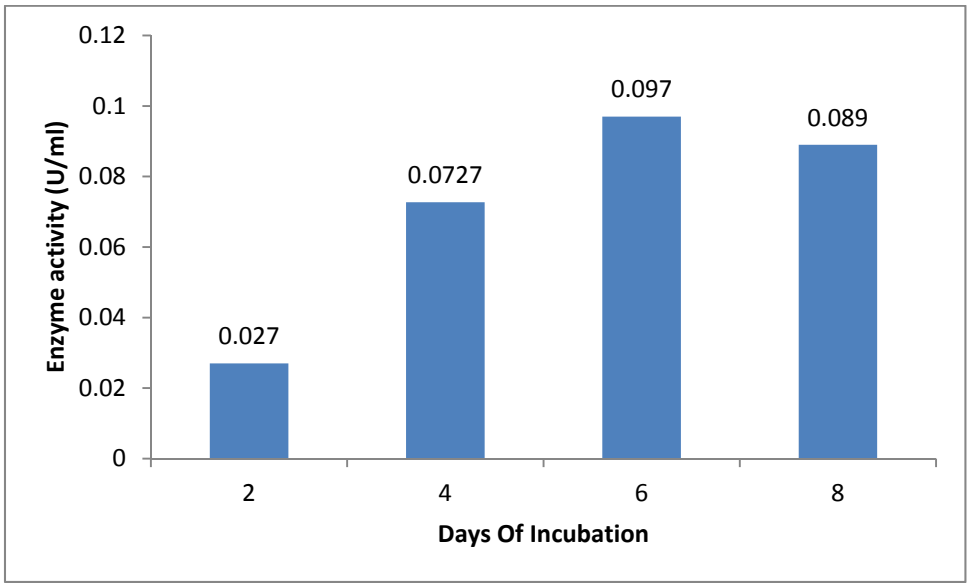


Figure10: Days of Incubation

3.4.2 Effect of Substrate Concentration

The substrate concentration was optimized by varying the concentration of CMC (0.2, 0.4, 0.6, 0.8 and 1.0 %). Results of the process showed that 0.8 % of substrate concentration provides maximum enzyme activity.

Substrate Concentration	Enzyme activity (U/ml)
0.2	0.075
0.4	0.025
0.6	0.11
0.8	0.16
1.0	0.16

Table 5: Substrate Concentration.

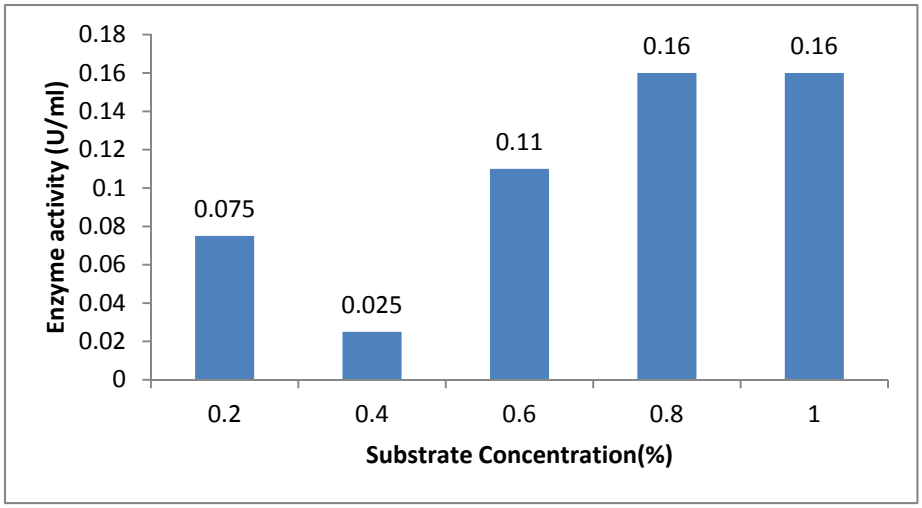


Figure 11: Substrate concentration

3.4.3 Effect of Carbon Source

The carbon source was optimized by varying the carbon sources (Fructose, Starch, Sucrose and Xylose). Results of the process showed that High cellulase production was obtained using xylose as carbon source.

Carbon Source	Enzyme activity (U/ml)
Fructose	0.401
Starch	0.103
Sucrose	0.509
Xylose	0.858

Table 6: Effect of Carbon Source

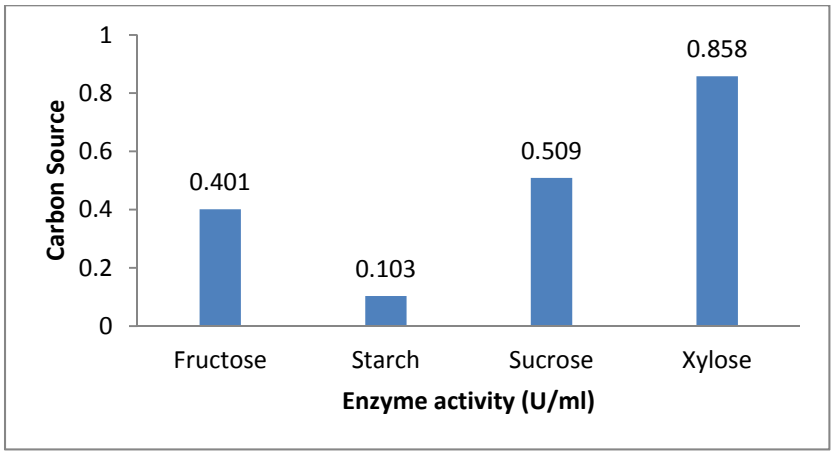


Figure 12: Effect of Carbon Source

3.4.4 Effect of Nitrogen Source

The nitrogen source was optimized by varying the nitrogen sources (Casein, Yeast extract, Peptone, Urea). Results of the process showed that High cellulase production was obtained using urea as nitrogen source.

Nitrogen Source	Enzyme activity (U/ml)
Casein	0.089
Peptone	0.074
Yeast Extract	0.087
Urea	0.113

Table 7: Effect of Nitrogen Source

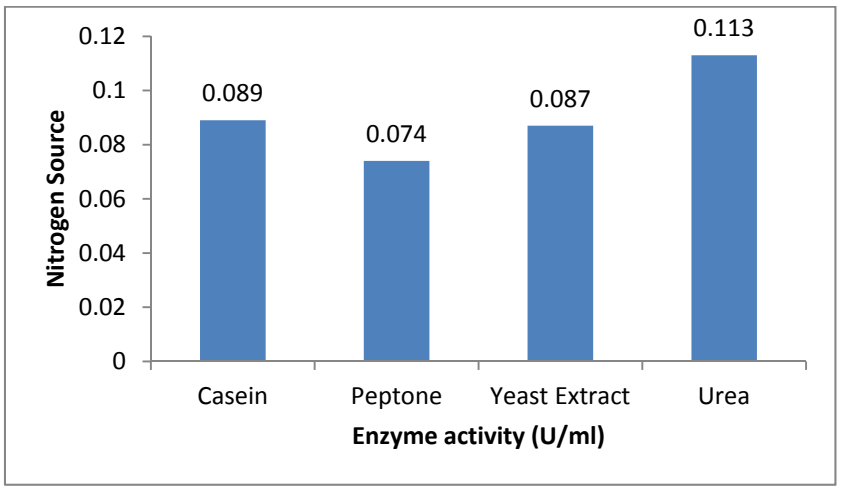


Figure 13: Effect of Nitrogen Source

3.4.5 Effect of pH

The pH was optimized by varying the acids and bases (5, 6, 7 and 8). Results of the process showed that pH-5 provides maximum enzyme activity.

pH	Enzyme activity (U/ml)
5	0.078
6	0.05
7	0.068
8	0.06

Table 1: Effect of pH

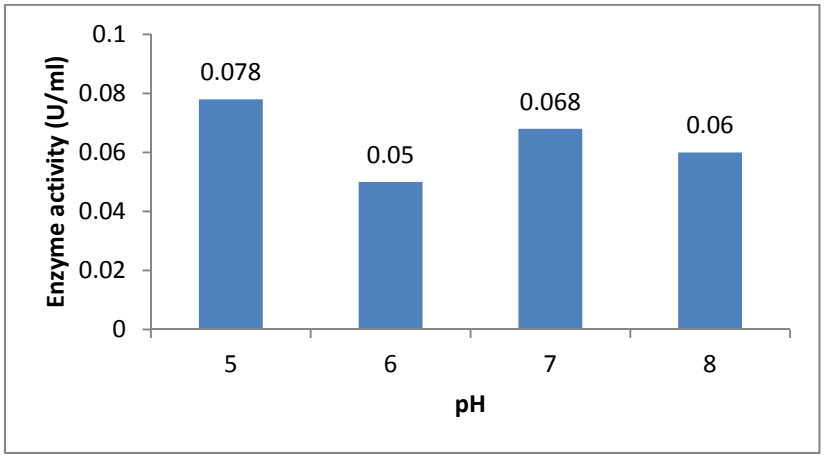


Figure 14: Effect of pH

3.4.6 Effect of Temperature

Incubation temperature plays an important and significant role in the metabolic activities of microorganisms. It was observed that enzyme activity was high in the flasks that were kept in incubator compared to that kept in refrigerator, room temperature and hot air oven.

Temperature	**Enzyme activity (U/ml)**
Refrigerator(8°C)	0.024
Room Temperature(25°C)	0.043
Incubator(45°C)	0.054
Hot Air Oven(65°C)	0.034

Table 2: Effect of Temperature

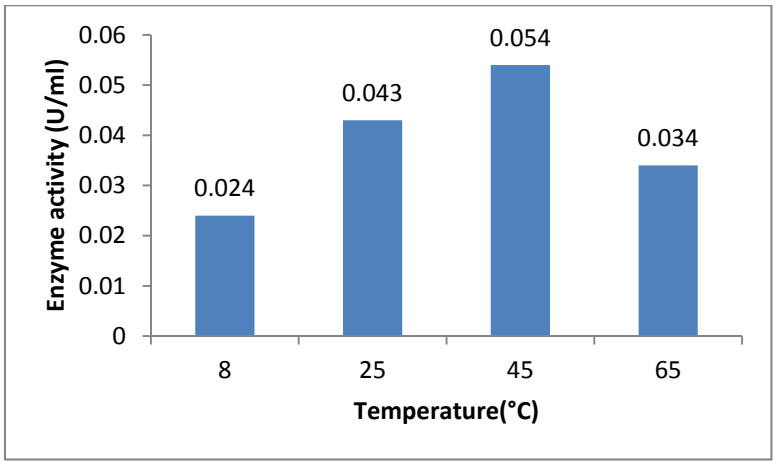


Figure15: Effect of Temperature

Purification and Characterization of cellulase

Preparation of enzyme for purification (Dialysis)

The proteins in the supernatant were precipitated with 75% ammonium sulphate saturation. All the subsequent purification steps were carried out at 4°C. The protein was dissolved in 0.1M sodium acetate buffer, pH 5.8 and dialyzed against the same buffer.

Estimation of protein content

SNO	Sample	Protein Concentration (mg/ml)	
		CRUDE ENZYME	AFTER DIALYSIS
1	SV	0.84	1.04

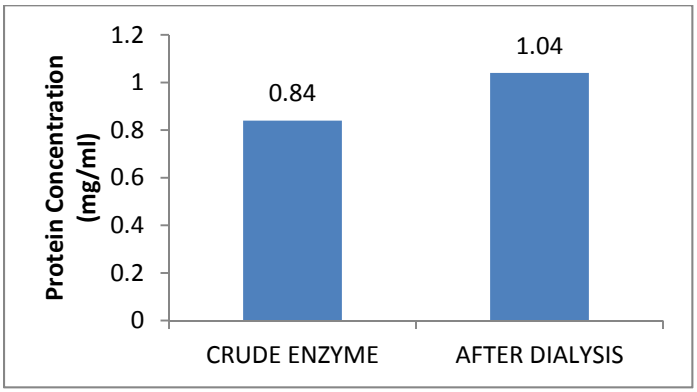


Figure15: Estimation of protein content

Estimation of enzyme activity

SNO	Sample	Enzyme activity(U/ml)	
		CRUDE ENZYME	AFTER DIALYSIS
1	SV	0.169	0.287

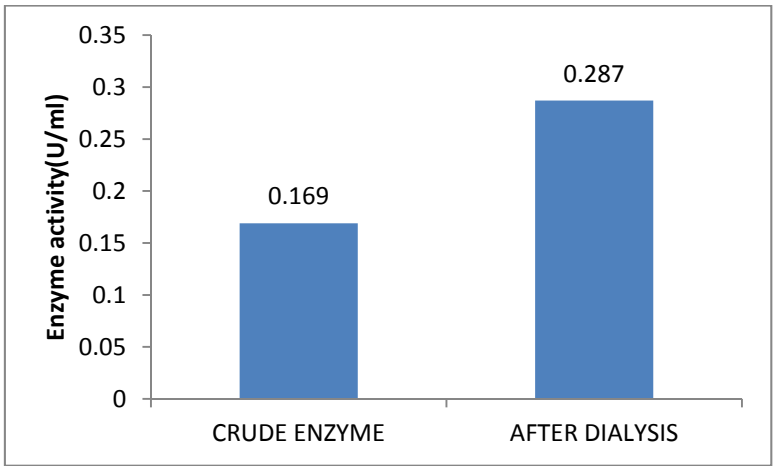


Figure 16: Estimation of enzyme activity

Electrophoretic studies - Extracellular polypeptide profile on SDS PAGE

The dialyzed protein sample of the cellulose enzyme was analyzed on SDS-PAGE and stained with CBB. Multiple bands were seen indicating the dialyzed samples were electrophoretically not homogenous.

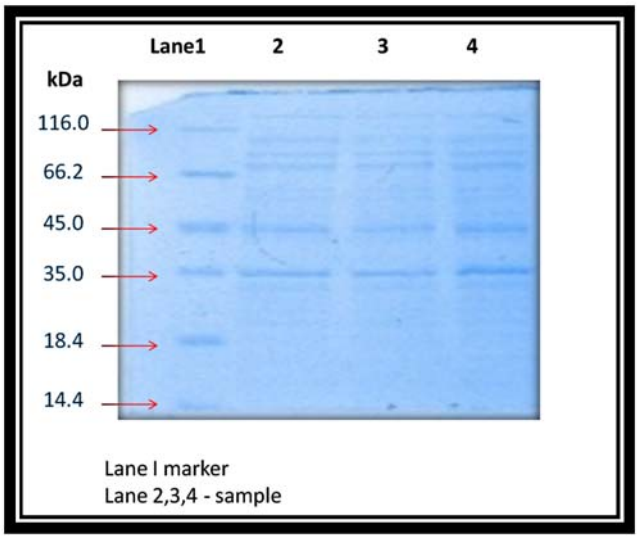


Figure:17: SDS-PAGE

Purified cellulase activity on Native PAGE

The activity of purified cellulase with crude positive control was determined on native PAGE, that appeared as clear dark brown bands.

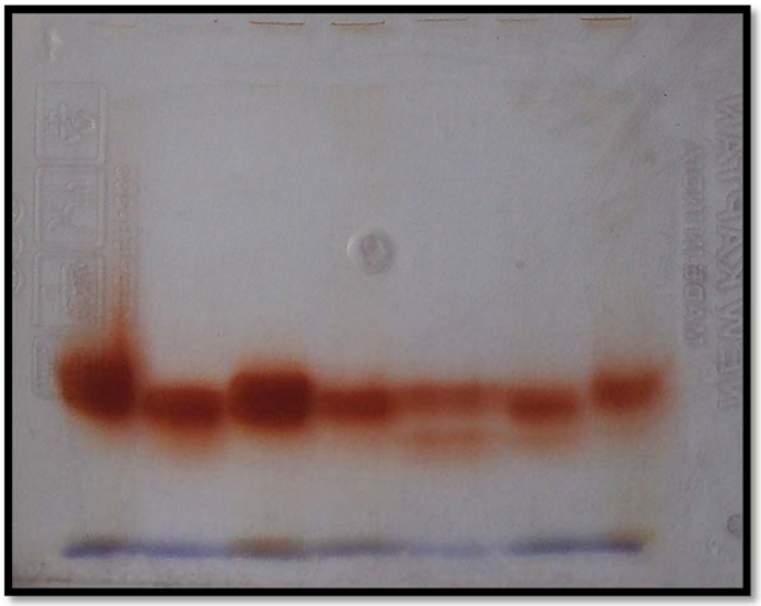


Figure:18: Negative PAGE

Lane 1 – 7: Dialysed crude cellulose

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