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A STUDY ON CMC HYDROLYSING NATURE OF *BACILLUS SUBTILIS*

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ABSTRACT

19 Bacterial isolates were obtained from soil samples, and screened for Cellulase production. Screening was done on Minimal Agar Media and zone of hydrolysis as obtain by performing Congo Red Test and Destaining by 1N NaCl. Staining and Biochemical characterization of selected isolates revealed it as Gram positive, rod shape and cellular arrangement was streptobacillus later identified as *Bacillus subtilis*. Growth of the isolate was found to be maximum at 37°c and pH 7.

Key words: Bacterial activity, Antibiogram analysis, Agar well diffusion, Solvent extraction

INTRODUCTION

An enzyme is a protein formed by the living organisms that acts as the catalyst to carry out certain desired reaction. Enzymes are very specific in their action. Each enzyme is designed to carry out a specific action with a specific response. They are highly selective catalysts, greatly accelerating both the rate and specificity of metabolic reactions, from the digestion of food to the DNA synthesis. In the enzymatic reaction the molecule at the beginning of the process is called as substrates, are converted into different molecules called as products. Almost all chemical reaction in the biological world needs enzymes in order to occur at the rates sufficient for life

In 1833, French chemist **Anselmepayen** discovered the first enzyme, diastases in 1877, German physiologist **Wilhelm kuhne (1837-1900)** first used the term enzyme, which comes from Greek origin means "in leaven". Enzymes in large globular proteins and range from just 62 amino acids residues in size to over 2,500 residues in animal fatty acid synthesise.

Most enzymes are much larger than their substrate they act upon and only a small portion of the enzyme is directly involved in catalysis. The region that contains the catalytic residues binds the substrate and then carries out reaction known as active site. Like proteins enzymes are also long linear chains if amino acids that fold to produce three dimensional products.

As the enzyme's name is often derived from its substrate or the reaction it catalyses, with word ending in –ASE for instance cellulases. On that bases international union of biochemistry and molecular biology have classified enzymes as oxidoreductases, Transferases, Hydrolases, Isomerases and Ligases.

Cellulose is a linear polysaccharide of glucose residues connected by β 1, 4 linkages like chitin, it is not cross linked having crystalline nature. Cellulose is insoluble and occurs as fibres of high density. Its density and its complexity make it resistant to the hydrolysis without preliminary chemical or mechanical degradation or swelling. In nature cellulose is usually associated with other polysaccharides such as xylan or lignin. It is sketal basis of cell wall and according to the **Spano, *et al.*, 1975.**

Cellulose is the most abundant organic source of food fuel and chemicals although its usefulness is dependent on its hydrolysis to glucose. Celluloses are distributed throughout the biosphere. Cellulose, a polymer of glucose, is the primary structural component of most plant cell walls. This polysaccharide is the most common carbohydrate on earth, relatively few animals are able to utilize this resource efficiently. Cellulose is the most common organic polymer, representing about $1.5x10^{12}$ tons of the total annual biomass production through photosynthesis, and is considered to be an almost inexhaustible source of raw material for different products. It is the most abundant and renewable biopolymer on earth and the dominating waste material from agriculture The basic structure of cellulose are 1, 4-β-glycosidic linked D-glucose molecules that form un-branched chains, consisting of several thousands of glucose molecules. The number of glucose units in the cellulose molecules varies and the degree of polymerization ranges from 250 to well over 10000, depending on the source and treatment method. Though lignocellulosic biomass is generally recalcitrant to microbial action, suitable pre-treatments resulting in the disruption of lignin structure and increase accessibility of enzymes have been shown to increase the rate of its biodegradation. In nature, cellulose is present in nearly pure state in a few instances, while in most cases the cellulose fibres are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin Cellulolytic microbes are primarily carbohydrate degraders and are generally unable to use proteins or lipids as energy sources for growth **(Sukumaran, *et al.*, 2005*).***

Cellulose is a crystalline polymer, an unusual feature among biopolymers. Cellulose chains in the crystals are stiffened by inter and intra chain hydrogen bonds and the adjacent sheets which overlie one another are held by weak Van-der Waals forces. The result of this process is a crystalline structure which is very difficult to degrade. However, cellulose fibres are not completely crystalline, but contain several types of irregularities, such as micropores or kinks, with amorphous character. These regions are the points of attack for the cellulolytic enzymes. When the cellulolytic enzymes cleave the glucose chains, the results are called cellodextrins, which are short glucose chains of various lengths.

The shortest ones, i.e. glucose dimmers, are called cellobiose and are sometimes not included in the cellodextrin classification). Hemicellulose is a mixture of short linear and branched polymers consisting of different pentose and hexose sugars. 20-35% of the plant dry weight consists of hemicellulose, thus it is the second most abundant polymer in the world**).** It mostly consists of xylose, a pentose sugar, which implies a problem for bioethanol production, because not all microbes can metabolize xylose. If it is not possible to overcome this problem, a large fraction of the sugars in the cellulosic biomass will not be available for fermentation **(Goldschmidt, 2008)**

Utilization of cellulose as a nutrient source requires enzymes that cleave beta- 1, 4 glycosidic bonds between constituent sugars. The enzymes referred to as cellulases, are require to split beta- 1, 4 glycosidic bonds in the polymer to release glucose units. The processes are called as cellulolysis. A large number of microorganisms are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of cell- free enzymes capable that of completely hydrolysing crystalline cellulose in vitro.

Cellulases have been the target of active research for over five decades, and are currently the third largest industrial enzyme worldwide (by dollar volume) because of their use in cotton processing, paper recycling, as detergent enzymes, in juice extraction, and as animal feed additives**.** Thus, if ethanol (or another fermentation product of sugars), produced from biomass by enzymes, becomes a major transportation fuel, cellulases will become the largest volume industrial enzyme **(Wilson, 2009).**

The enzymatic hydrolysis of cellulose releases soluble sugars including glucose, xylose, and other hexoses and pentoses. To make the sugar monomers available for fermentation, the cellulose and hemicellulose chains have to be hydrolyzed. The hydrolysis of hemicelluloses are catalyzed by xylanases, together with other accessory enzymes (α-L-arabinofuranosidases, feruloyl and acetyl xylanesterases, β-xylosidases, etc.), while the hydrolysis of cellulose can be undertaken by microorganisms that produce enzymes known as the cellulase systems **(Gusakov, *et al.,* 2007).**

METHODOLOGY

Sample Collection:

Five different samples were collected from different location in Lucknow for our project work given below is the table which gives the information regarding the location and the type of soil samples we collected.

Table 1: List of all the Samples we Collected During Our Work

Sample No.	Sample type	Location
First	cow dung soil	Gomtinagar railway station lucknow U.P.
Second	Forest soil	Dehradun forests
Third	Decayed leaves	Gomtinagar railway station lucknow U.P.
fourth	Paper wastes	Lucknow U.P.
Fifth	Cow dung	Lucknow U.P.

Isolation of bacteria from soil sample:

The samples were serially diluted in 0.85% NaCl solution and spread over sterilized nutrient agar media. Further the samples selected on the basis of their morphology and then pure cultures were prepared by using streaking technique.

Screening of cellulase producing bacteria:

The cultures were restreaked in sterilized minimal salt agar media supplemented with 1% CMC and then incubated at 37°C for 48 hours. Positive cultures were selected on the basis of clear zone of hydrolysis, after washing with 1M NaCl solution.

Optimization of production media:

The each component of the fermentation media was optimized on the basis of OFAT rule.

Fermentation and downstream processing:

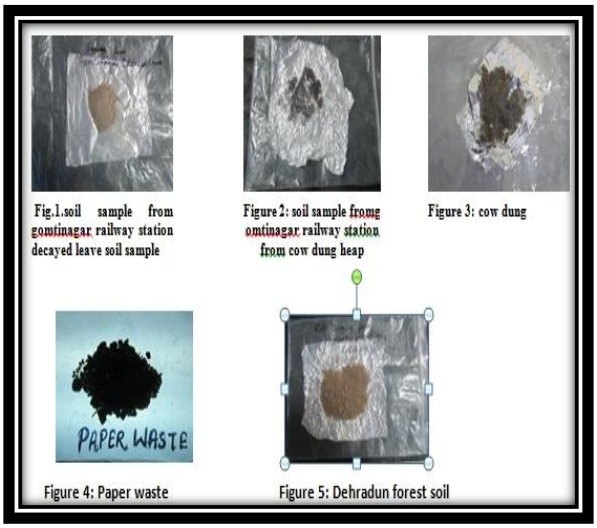
The cellulase production were carried on sterilized optimized fermentative media and incubated for 48 hours. The enzyme was purified by salt precipitation and dialysis.

Estimation of cellulase enzyme:

The estimation of cellulase was carried by Lowry's estimation and DNS assay.

RESULTS

Soil samples were collected from different locations which possible have the cellulolytic activity. At least five different soil samples were examined for the cellulolytic activity. Following are the pics of the soil samples which we collected under sterilize conditions.



Isolation of the microbes by serial dilution and agar plating method

Microbes from the soil samples were isolated by serial dilution and NA plating technique. Mixed cultures were obtained after spreading as shown below in the figs. below.

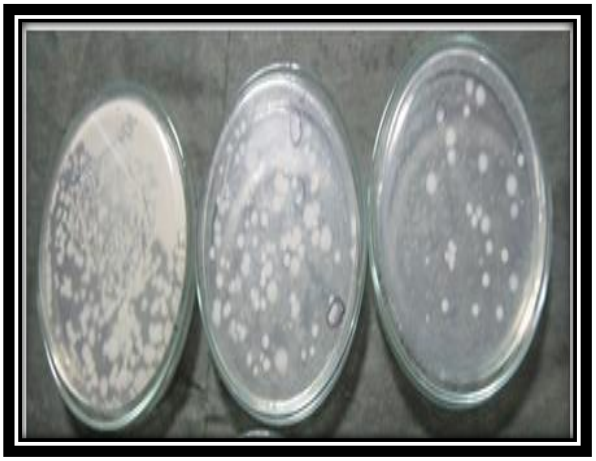


Figure 6: Mixed cullture plates of first soil sample

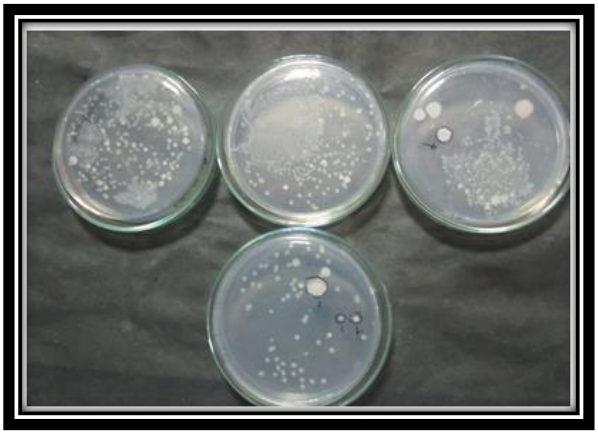


Figure 7: Mixed cultures of the dehradhun soil sample

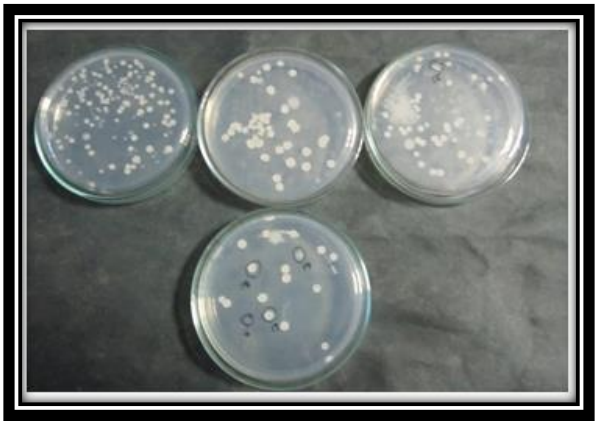


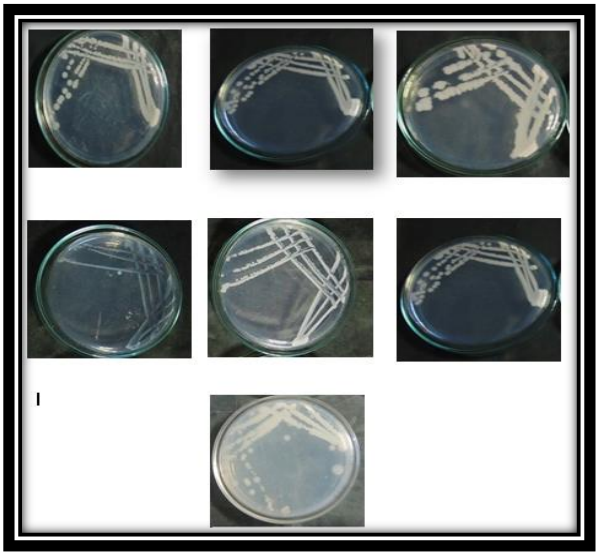
Figure 10: Mix culture plates of the fifth sample

Colony morphology

The cultures were obtained were further differentiated on the basis of their morphology .The physical characteristics of the colonies such as colony SHAPE, MARGIN ELEVATION, SURFACE, PIGMENTATION, TEXTURE and OPACITY of each colony obtained in the mixed culture were studied. It helped us to select the different colonies on the basis of the physical characteristics. The morphology of all the selected colonies is given in the table below.

Pure cultures

Pure cultures were obtained after discontinuous quadrant streaking of the colonies selected from the mix culture on the NA plates. The figures of all the pure cultures are given below.



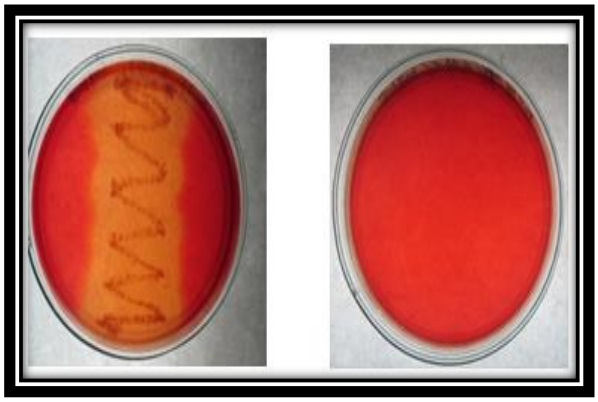
Screening of pure cultures

The pure cultures we obtained were screened for the cellulases activity.MAM media (180ml) was prepared supplemented with 1% CMC autoclaved and poured into the sterilised plates ant allowed to solidify. After that the cultures were centrally streaked in the solidified MAM media plates. Plates were incubated at 37 ºC for 48 hirsute check the potential activity of the cultures the plates were subjected to the secondary screening, plates were flooded with 1% Congo red and then distained with the 1MNaCl.after the distained process the plates were examined for the core zone of hydrolysis. The **Table-3** shows the remarks of the cultures after the secondary screening. Secondary results showed that the culture no.MJLY1403 shows the maximum core zone of hydrolysis.

Table 3: Results of Screening of Purified Plates

SERIAL NO.	CULTURE NAME	REMARKS
01	MJLY1401	-
02	MJLY1402	++
03	MJLY1403	+++
04	MJLY1404	-
05	MJLY1405	-
06	MJLY1406	-
07	MJLY1407	++
08	MJLY1408	++
09	MJLY1409	+
10	MJLY1410	+
11	MJLY1411	-
12	MJLY1412	-
13	MJLY1413	-
14	MJLY1414	+
15	MJLY1415	-
16	MJLY1416	+
17	MJLY1417	+
18	MJLY1418	+
19	MJLY1419	+

Secondary screening results of all the cultures



Effect of different physiochemical conditioned on isolate MJLY1403

The isolate selected for the further work was subjected to different physiochemical condition to check the best conditions for the growth of the isolate bacterial

Effect of Temperature on the Isolate MJLY1403

The culture MJLY1403was streaked on four NA plates and all the four plates were kept at four different temperatures such as 10°C, 33°C, 37°C and 55°C. **Table -4** shows the results of the effect of the temperature on the culture noMJLY1403.

Table 4: Effect of Temperature on Culture no MJLY1403

S. No.	Temperature	Remarks
1.	10° C	-
2.	33° C	++
3.	37° C	+++
4.	55° C	-

Table5: Shows the Remarks and Results of Temperature Effect on MJLY1403

S No	Remarks	Results
01	-	No growth
02	+	Little growth
03	++	Normal growth
04	+++	Maximum growth

Effect of pH on the isolate MJLY1403

The isolate MJLY1403 was inoculated in the seven NA flasks with different ph as 5, 5.5, 6, 6.5, 7, 9 and 11. After the completion of the incubation period the O.D was taken given in the Table 5. The results showed that tour culture shows maximum growth at ph 6.esults mentioned below.

Table 5: Effect of pH on Culture No MJLY1403

S NO.	pH	O.D.at 600nm
01.	5.0	1.09
02.	5.5	1.18
03.	6.0	1.48
04.	6.5	1.44
05.	7.0	1.28
06.	9.0	1.10
07.	11	0.1

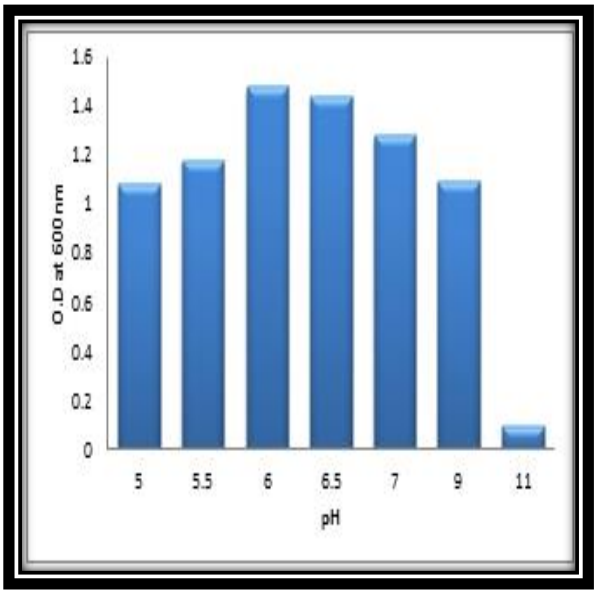


Figure 36: Effect of pH on growth of MJLY1403

Identification of the bacterial culture

From the screening results we concluded that the culture No.MJLY1403 have the maximum cellulolytic activity further the culture was identified by different techniques in accordance with Bergey's manual mentioned below.

Table 6: Staining and Biochemical Tests of MJLY1403

Test	Result
Gram staining	+ve, Bacillus
Catalase test	+ve
Mannitol test	+ve
MR VP test	-ve
ENDOSPORE TEST	+ve

From all the results we did in accordance to the Bergey's manual the Bacteria was identified as *Bacillus subtilis.*

Study of the growth curve parameters of culture MJLY1403

We inoculated our culture MJLY1403 in the NB medium and the O.D was taken after every 24hrs for consecutive seven days using calorimeter. Table 7 shows the date wise O.D of our culture.

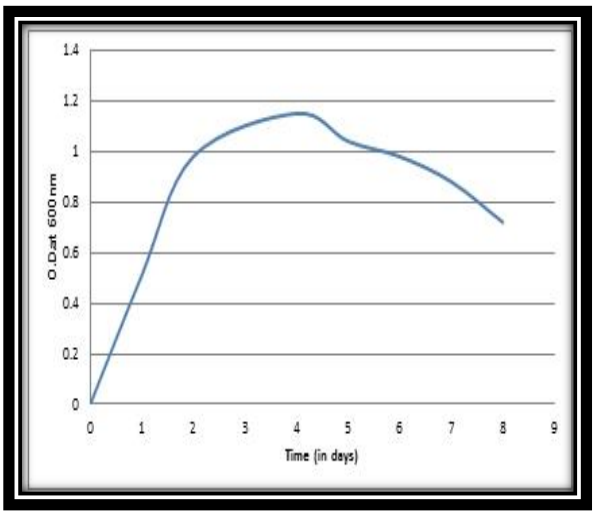


Figure 8: Growth curve study

DNS ASSAY

A standard graph was plotted to calculate the enzyme activity. By the help of the standard graph we can calculate the amount of glucose released by comparing the O.D. Following Table 7 shows the reading of the different concentration of the glucose.

Optimization of incubation time for the best enzyme activity

The culture no MJLY1403 was inoculated in the production media and after every 24hrs the O.D was taken to find out the amount of the glucose released. Hence gives the complete information regarding the activity of our bacterial isolate.

The standard graph plotted earlier was used to calculate the enzyme activity by comparing the O.D. The tangents were drawn on the Y axis related to the absorbance reading given by the calorimeter, from that point another line was drawn to the X axis which gives us the concentration of the glucose released (mg/ml) per day thus the enzyme activity can be calculated.

Optimization of production media

The production media we used was optimized according to the need of our culture so as to get the best results of the growth and the enzyme activity. The different physical and the chemical factors of the production were changed such as the nitrogen source, pH and the substrate concentration, were varied and the O.D was taken from the second day after every 48hrs the O.D was compared with standard graph and from the results we got best nitrogen sources, pH and the substrate concentration for our bacterial strain.

Effect of different nitrogen sources

We varied the production media by testing the different nitrogen sources. And from the results which we obtained after the DNS assay we concluded that the combination of the yeast extract and the beef extract are the best nitrogen sources for our bacterial culture. Table 08 shows the O.D measured and the activity of the bacterial culture.

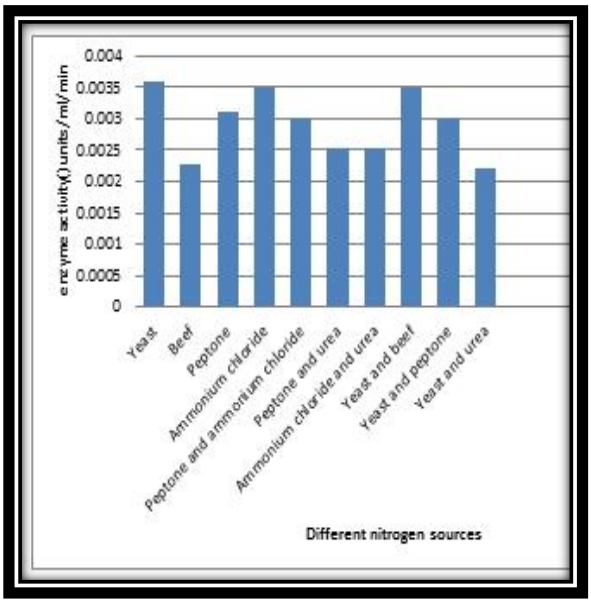


Figure 41: Different Nitrogen Sources

Effect of the Different Concentration of the Beef and Yeast Extract

After changing the nitrogen sources in the production media.The concentration of the selected nitrogen source was altered to get the best results. After the DNS assay the best concentration was selected as 1.5% of the beef and yeast extract .Table 11 shows the O.D measured and the activity of the enzyme.

Table11: Effect of the Different Concentration of the Yeast and Beef Extract

Different conc. Beef and yeast extract (in %)	O.D (540nm)	Glucose released (mg/ml)	Enzyme activity (units/mg/ml)
.1	0.19	0.07	0.00168
0.5	0.17	0.125	0.003
1.0	0.14	0.105	0.00252
1.5	0.20	0.15	0.0036
2.0	0.18	0.13	0.00312

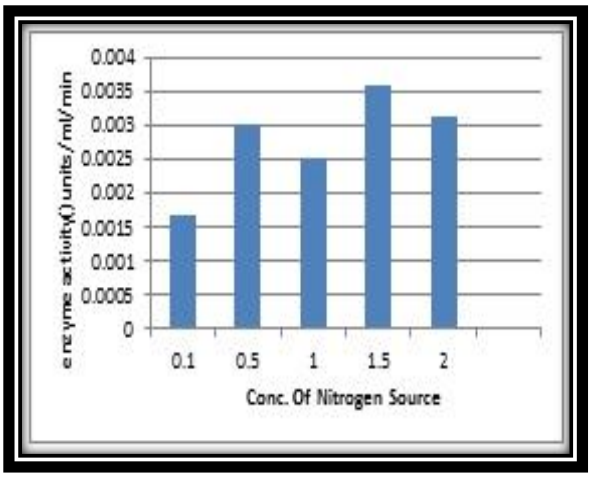


Figure 42: Effect of Different Concentration of Nitrogen Sources

Effect of the different substrate concentration on the enzyme activity

Substrate concentration in the production media was also changed and it was concluded by the help of the DNS assay that the substrate concentration of 1.5% is best for our isolate Table 12 below shows the enzyme activities at the different substrate concentrations.

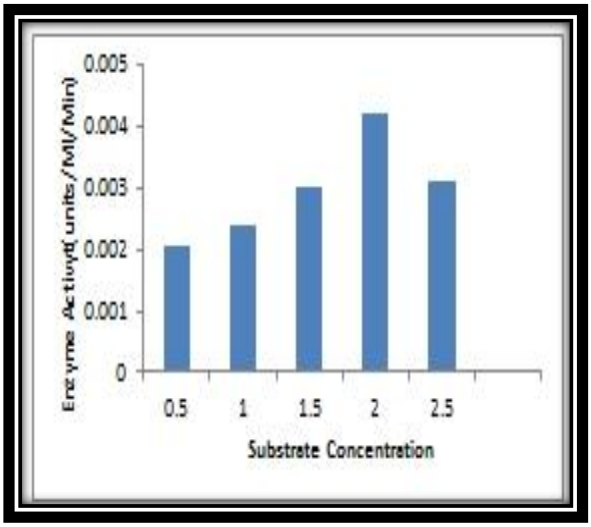


Figure 43 Effect of Substrate Concentration on Enzyme Activity

Fermentation of MJLY1403

Fermentation was done by using shake flask method. The bacterial strain was fermented in the optimized media under optimum conditions for five days. After that the crude enzyme extract by using centrifugation technique.

Assessment the enzyme activity during fermentations by DNS assay

The DNS assay of the fermentation broth was done on the fourth, fifth, sixth day.

Table 13: DNS assay during Fermentation

Time(in days)	O.D	Glucose released(mg/ml)	Enzyme activity (units/ml/min)
4	0.26	0.18	0.00432
5	0.29	0.20	0.0048
6	0.31	0.215	0.00516

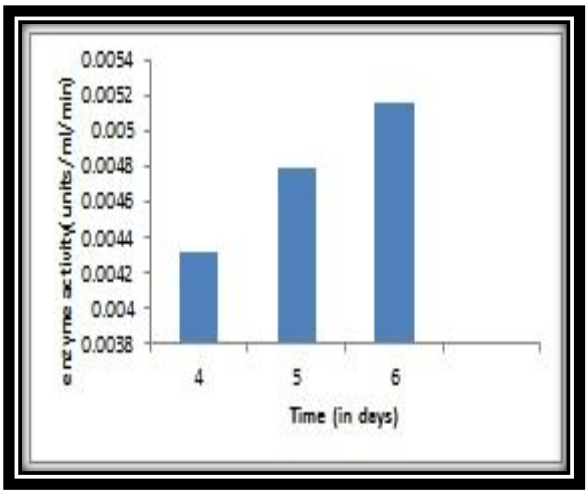


Figure 44: Effect of Incubation Time on Enzyme Activity

Extraction of the crude enzyme after fermentations

After the fermentation the culture media was centrifuged and the supernatant was collected and stored at 4 ºC. Total supernatant we collected was measured as 80ml.

Salt precipitation

The crude enzyme we obtained after the centrifugation was salt precipitated under cold conditions. After the completion of the salt precipitation the salt precipitated enzyme was centrifuged and the pellet was dissolved in the Tris buffer of pH6. 20ml of the enzyme in the Tris buffer was obtained.

Dialysis

Dialysis was done in order to remove the salts present in the salt precipitated enzyme

Effect of pH on Enzyme Activity

DNS assay of the purified enzyme was done by using the Tris of the different pH as 5, 6, 7, 9 and 11 and O.D was read and the activity at the different pH was calculated. Table 14 shows the O.D and the activity of the enzyme at the different pH.

Table 14: Effect of Different pH On the Enzyme Activity

S No.	Different pH	O.D(540nm)	Glucose released (mg/ml)	Enzyme activity (units/ml/min)
01	5	0.16	0.115	0.00276
02	6	0.18	0.13	0.00312
03	7	0.10	0.075	0.0018
04	9	0.16	0.115	0.00276
05	11	0.18	0.13	0.00312

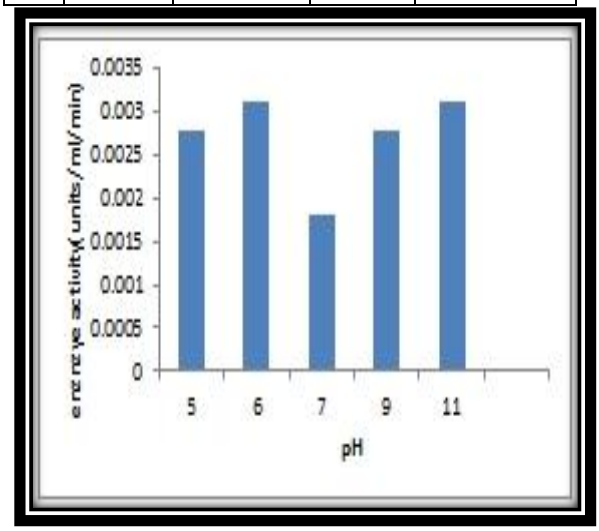


Figure 45 Effect of pH on Enzyme Activity

Effect of different temperature on the enzyme activity

To check the activity of the enzyme at the different temperature the enzyme with the substrate were incubated at different temperatures like 10^0c, room temp.37 ºC, 55ºC, 65 °C, 75 ºC. Table 11 Shows the O.D and enzyme activity at the different pH at 540nm.

Figure 43 Effect of temperature on enzyme activity

Different Temperature(°C)	O.D (540nm)	Glucose Released (mg/ml)	Enzyme activity (units/ml/min)
10	0.20	0.15	0.0036
33	0.16	0.115	0.00276
37	0.17	0.125	0.003
55	0.21	0.15	0.0036
65	0.20	0.15	0.0036
75	0.17	0.125	0.003

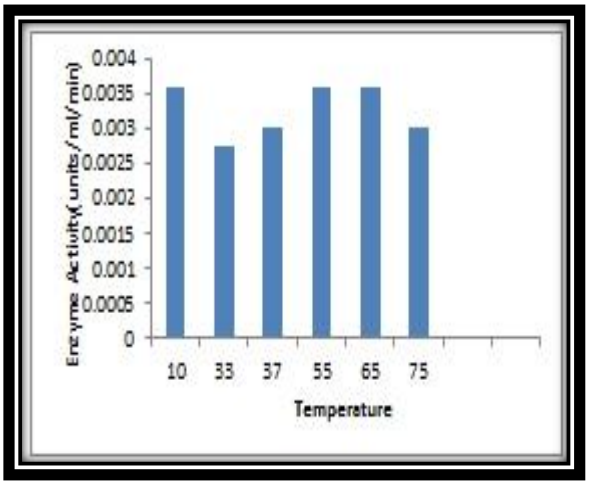


Figure 46: Effect of Temperature on Enzyme Activity

LOWRY'S ASSAY

To estimate the protein content in the sample standard graph was prepared by using the known protein of known concentration. Reading of the sample we compared with standard graph and the content was estimated. After plotting the standard graph the Lowry's assay was done on the crude salt precipitated and the dialyzed protein. The readings were compared with the Lowry's standard graph and the total protein content was determined.

Table 19: Shows Protein Content

S No	Enzyme type	O.D at 660nm	Protein conc (mg/ml)
01	Crude enzyme	1.20	1.70
02	Pure	0.44	0.60

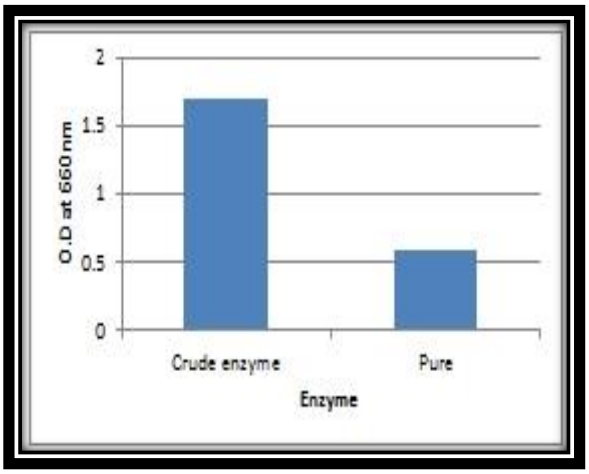


Figure 48: Protein Concentration of Crude and Salt Precipitated Enzyme

DNS Assay of the Enzyme

The DNS assay was done in order to find out the activity of the different forms of the enzyme like crude salt precipitated and the pure enzyme. The reading was compared with the standard graph and the activity of the enzyme was calculated. Table 15 shows the O.D and the enzyme activities.

Table 20: O.D and Enzyme Activity

Enzyme used	O.D at 540nm	Glucose released	Enzyme activity (units/ml/min)
Pure enzyme	0.27	0.19	0.00456

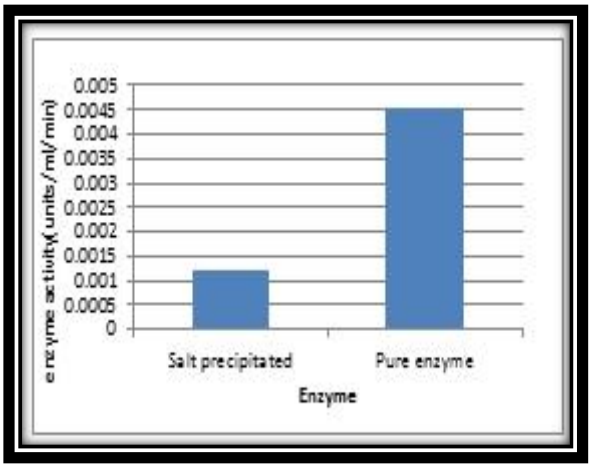


Figure 43: DNS Assay of the Pure and Salt Precipitated Enzyme

DISSCUSION

Microorganisms were isolated from the soil by serial dilution and agar plating method as earlier done by the **[4].** isolates were purified which were named as MJLY1401-MJLY1419**.**

The culture were grown minimal agar medium (pH 7) supplemented with 1%CMC and then plates were screened for the cellulolytic activity as earlier done by the **[7].** By flooding the plates the the Congo red.MJLY1403 showed the largest zone of the hydrolysis was selected as previously done by morphological properties and the taxonomical characteristics of the isolate were studied according to the Bergey's manual as earlier done by the Kanmani et al., (2011) and the isolate were identified as *Bacillus subtilis.*

Production media for the cellulose production was optimized according to the isolate requirements. The optimized media contains 2%CMC, 0.75% beef extract, 0.75% of yeast extract, $MgSo_4$, KH_2PO_4, K_2HPO_4 sodium citrate as previously done by Verma *et al* (2012). partial purification was done by the ammonium salt precipitation .ammonium salt was added to the supernatant to bring saturation in the cold conditions and then the suspension was dialysed against 100mMTris of pH6 as done earlier by Vipul V *et al* (2012) followed by the dialysis

The enzyme activity was assayed by incubating the 0.5ml of enzyme with 0.5ml of 1%CMCin the100mm Tris buffer pH6 after the incubation at the 37°C for 15mins the reaction was stopped by adding 1ml of DNS reagent and the reducing sugars were assayed calorimetrically as done earlier by (Yin., *et al* 2010). Protein concentration was measured by Lowry's method using BSA as done earlier by (Yin., *et al .*,2012). The activity of purified enzyme was calculated to be 0.00456 which is comparable to the cellulase purified earlier.

SUMMARY AND CONCLUSION

This period of the project work includes the isolation ad bacteria from soil by serial, dilution and agar plating method. Screening of the isolate for the production of the cellulases, study of the growth curve parameters, of +ve isolate, optimization of the media for the optimum production of the cellulase ,physiochemical conditions were optimized so to favour isolate conditions, ammonium salt precipitation followed by the dialysis. On the basis of these results the conclusion can be drawn which are as follows.

Among the isolates the isolate MJLY1403is identified as the best isolate for the production of the cellulases. The growth of the isolate was found to best at the pH6 and at the temperature of 37°C

After the optimization of the production media as, 0.75% yeast extract, 0.75% beef extract and 2% of CMC of the substrate concentration was found to best for our isolate MJLY1403 Crude extract obtained was partially purified by the salt precipitation and followed by the dialysis. Further work includes purification of the enzyme by the help of more sophisticated technique such as ion exchange chromatography, affinity chromatography.

The molecular weight can be determined by SDS PAGE technique. Mutation can be caused for the better results

DNA recombination can be done as the bacteria which we use have 6 days life cycle. So as to decreases the expenses on the fermentation the bacterial gene responsible for the production of cellulases can transformed into other microbes which grows fast. The effect of this cellulase on the cellulosic material like newspaper, filter paper, cellulosic municipal wastes or industrial wastes could also be studied, which increase its application.

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