International Journal of Science and Research (IJSR)
ISSN: 2319-7064
ResearchGate Impact Factor (2018): 0.28 | SJIF (2018): 7.426

Comparative Studies on Enhanced the Production of Cellulase by Physical and Chemical Mutagens

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Abstract: ***Cellulose is abundantly present in plants sources. Breakdown of cellulose in glucose can be used in various applications. Breakdown of cellulose is done by cellulase. In this research article production of cellulase enzyme was increase by bacteria through the mutation and media optimization.***

Keywords: cellulose, cellulase, CMC, screening, mutant strain, strain improvement

1. Introduction

Cellulose is s structural compound. It is a polysaccharides, contains the cell wall of plants mainly in roots, stems,branches and all woody portion of plant cellulose is a fibrous compound it is totally absent in animal [1,2]. Cellulose is fibrous compound present in vegetables33%, cottons 90%, woods 50%. In other parts of sample plant, it is present as lignocellulose. Due to absence of hydrolase the cellulase which is responsible for cleave the β- linkages of cellulose [3]. In mammals, cellulose cannot be digested due to absence of hydrolase that cleaves the β- linkages of cellulose [4]. Herbivores can digest the cellulose as in their gut some microbes are present which possess the enzymes that can cleave β – glycosidic bond [5]. Cellulose is a linear un-branched homo-polymer. Cellulose is arranged parallel to each other and is joined together with hydrogen bond [6,7].

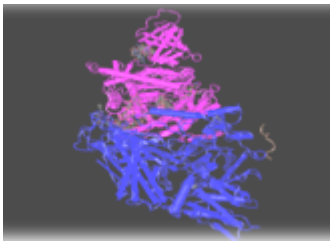

Figure 1: 3D structure of cellulose [8]

The cellulolytic bacteria sequence based on the 16S ribosome by CMC (carboxy-methyl cellulose) culture cellulolytic activity of bacteria on CMC culture by the clear zone [9]. The cellulolytic bacteria: -*Clostridiales, Cynobacteria, Actinobacteria* etc**.** The Cellulolytic bacteria secretes the free enzyme depends on the decompounds of lignocellulose into sugar by enzymes. Glycosidase hydrolyses help breakdown hemicelluloses. Cellulolytic bacteria are other resourcesfor cellulolytic enzyme [10]. Cellulose is enzyme which is produce by protozoans, bacteria and fungi. Cellulase will breakdown the polysaccharide sugars as cellulose into monosaccharide sugars or oligosaccharides [11,12]. Cellulase enzymes break the glycosidic bondss by the hydrolysis reaction [13]. Cellulose enzymes are of different types such as Endo 1-4, βglucanase, Exo β- (1,4) glucanases**,** β- d – glucosidase**,** Cellobiases**,** Carboxy-methycellulase, Cellulase A, Alkali cellulase [14].

2. Materials and Methods

2.1. Sample collection

The soil sample was collected near from the husk shop, Vishesh Khand, Gomtinagar, Lucknow.

2.2. Isolation of bacteria from sample:

The bacterial culture was isolated by serially diluting the soil sample in 0.85% sterilized saline and then the sample was spread on sterilized nutrient agar plates and incubated at 37°C for 24 hours [15].

2.3. Purification of bacteria from mixed culture plate

The bacterial colonies were streaked on sterilized nutrient agar plates by continuous quadrant method and then incubated at 37°C for 24 hours [16].

2.4. Screening for cellulase producing bacteria

The bacterial cultures from the streaked plates were re-streak in sterilized CMC (carboxymethyl cellulose) agar plates and then incubated at 37°C for 48-72 hours and then Congo-red test was performed[17,18].

2.5. Strain identification

For the identification of the strain the gram's staining, endospores staining, catalase test, mannitol test, MR-VP test was done.

2.6. Strain improvement by using physical and chemical mutations

(a)Physical mutation:
Physical mutation was performed to enhance the production cellulase. In thisthe UV exposure was given to the culture at different time intervals [19].

(b) Chemical mutation:
Chemical mutation was done to enhance the production of cellulase. Different concentration of ethidium bromide was used for chemical mutation [20].

2.7. Media selection and its optimization:

Table 1: Standard media was selected and optimization was done

S no.	Factors	Modified media	Standard media
1.	Production media	PM1	KH_2PO_4- 6g/ l
			Peptone- 5g/l
			$FeSO_4$- 0.2g/l
			CMC- 1%
			$MgSO_4$ - 0.2g/l
			Dextrose - 8g/l
		PM2	$(NH_4)_2SO_4$ - 1 g/l
			K_2HPO_4 -2 g/l
			$MgSO_4$- 0.2 g/l
			NaCl - 5 g/l
			CMC - 1 %
		PM3	KH_2PO_4- 2 g/l
			$FeSO_4$ - 0.2 g/l
			$CaCl_2$ – 0.2 g/l
			K_2HPO_4-2 g/l
			$(NH_4)_2SO_4$- 2 g/l
			$MgSO_4$- 0.2 g/l
			CMC- 1%
2.	Nitrogen source		
	Peptone	MM1	2g/l
	NH_4Cl	MM2	2g/l
	Malt	MM3	2g/l
3.	Carbon source		
	Maltose	MM4	5g/l
	Sucrose	MM5	5g/l
	Dextrose	MM6	5g/l
4	Different pH on the growth of the bacteria		
	pH	MM7	4
	pH	MM8	7
	pH	MM9	9
	pH	MM10	11

2.8. Bacterial growth study

The bacterial growth study was done at37°C at 1 hour of time interval. To check thebacterial growth kinetics [21].

2.9. Fermentation and downstream process

Fermentation was performed for the production of the cellulose in the selected optimized media by using shake flask method [22]. Further the purification was done by salt precipitation and dialysis [23].Fermented product was purified by salting out method by using 40 % ammonium sulphate salt. The enzyme assay was done by DNS test [24].

3. Results

3.1. Sample collection

The soil sample was collected after removing the upper layer of husk or straw materials form the shop.

Figure 2: The soil collected for the isolation of bacteria is in dark colour appearance.

3.2. Isolation of bacteria

The isolation of bacteria is done by serially diluting the soil sample in 0.85% NaCl solution and then spreading of the sample in nutrient agar plates.

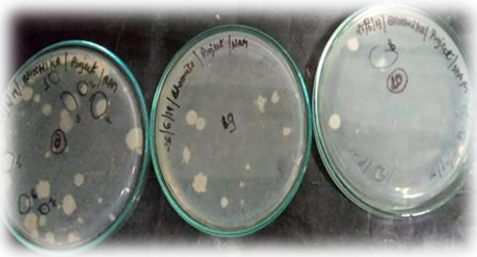

Figure 3: The isolated colonies after spreading

3.3. Purification of selected culture

The purification of the bacteria is done by streaking the selected colonies on the basis of different morphological parameters from mixed culture plate to pure culture plate.

Figure 4: The bacterial cultures after streaking by continuous quadrant method

3.4. Screening of pure cultures

For checking the production of the enzyme the isolated pure cultures were screened in the minimal salt agar media by adding the substrate of 1% CMC, and then Congo red test was performed.

Figure 5: The positive culture with clear zone in minimal salt agar media after Congo red test

3.5. Strain identification:-

The various biochemical tests were performed for the identification of the culture such as Gram's staining, Endospore staining, Catalase test etc.

Table 2: Biochemical test for bacterial identification

S no.	Biochemical Tests	Results
1	Gram's staining	Gram's positive &*Bacillus.*
2	Endospore staining	Endospore Positive
3	Catalse Test	Positive
4	Mannitol Test	Positive
5	VP Test	Negative

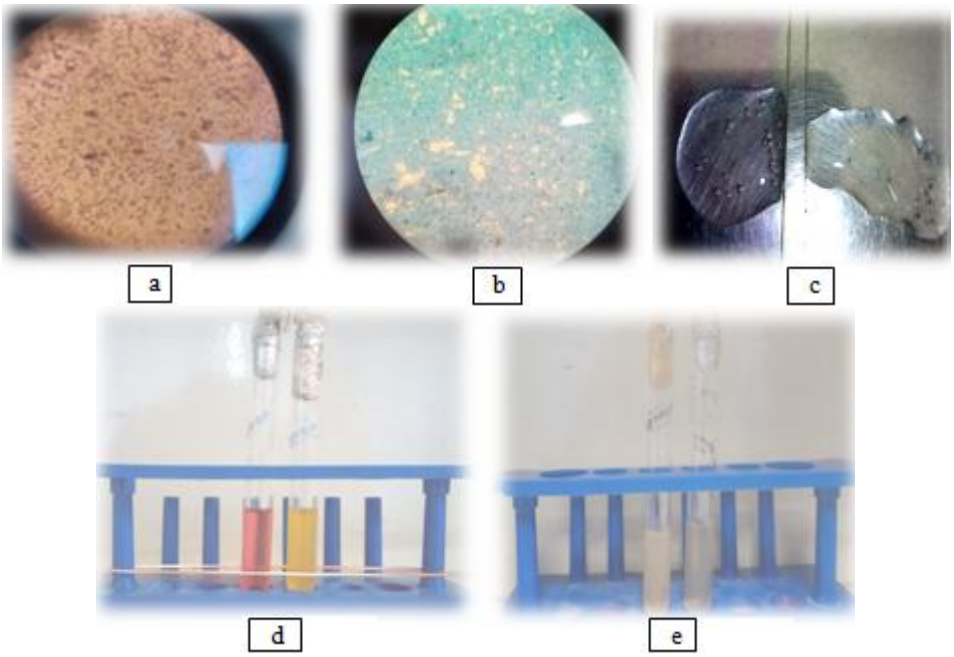

Figure 6: Representation of the results of biochemical tests a. Gram's Staining, b. Endospore staining, c. Catalase test, d. Mannitol test, e. VP test

3.6. Strain improvement:

The enhancement of the stain is done by the physical and chemical mutation.

Physical mutation:
The culture after spreading in nutrient agar plates was exposed to UV light at different time intervals. After the screening of bacteria, 2min UV exposure culture gave positive result.

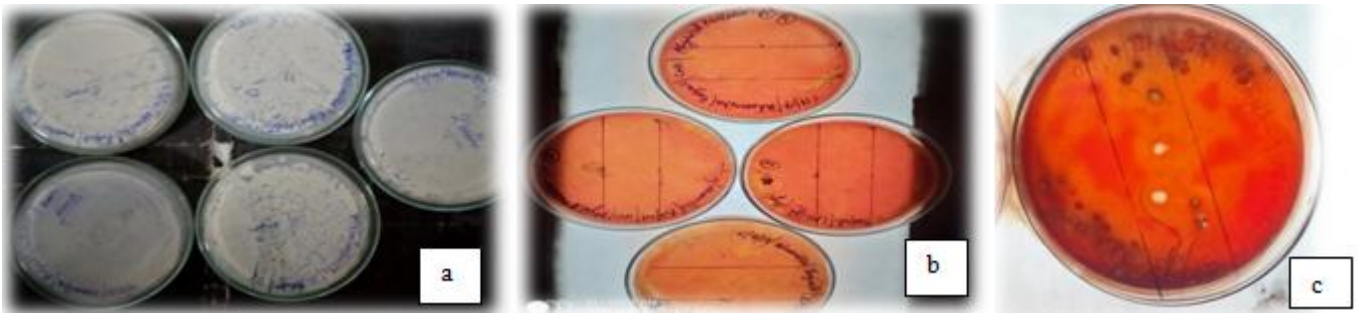

Figure 7: (a) Physical mutation through UV,(b) screening after mutation. (c) selected bacteria after mutation

Chemical mutation

The culture was inoculated in nutrient broth containing the different concentration of EtBr. By taking the OD at 620 nm, it was found that the culture in the presence of 3µl EtBr shows maximum growth, then the culture was spread and screened for the cellulose.

Figure 8: The plates showing the results of cellulase screening by EtBr mutation

3.7 Media selection and its optimization

The media selection and optimization is done for enhancing the growth of the wild strain, chemically mutated strain and physically mutated strain.

3.7.1 Media Selection

The growth of all the cultures was checked in three different production media. Where PM 1 was selected due to maximum culture growth.

Table 3: Media optimization for fermentation

S no.	Culture	OD (620nm)		
		PM1	PM2	PM3
1	Wild strain	0.22	0.04	0.05
2	Physical mutant	0.26	0.09	0.03
3	Chemical mutant	0.29	0.01	0.07

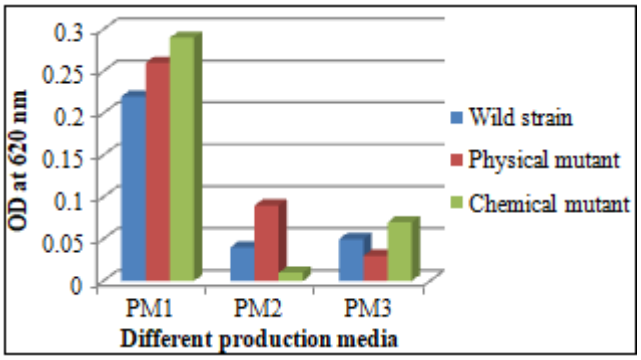

Figure 9: PM1 selected as best media

3.7.2 Optimization of selected media

The optimization of production media 2 was executed by changing the chemical parameters (Carbon and nitrogen sources) and physiochemical parameters (pH and temperature).

3.7.3 Effects of carbon sources

Table 4: Different carbon sources were used to find best carbon source

S no.	Culture	OD (620nm)		
		Dextrose	Sucrose	Maltose
1	Wild strain	0.10	0.06	0.04
2	Physical mutant	0.14	0.13	0.04
3	Chemical mutant	0.17	0.15	0.06

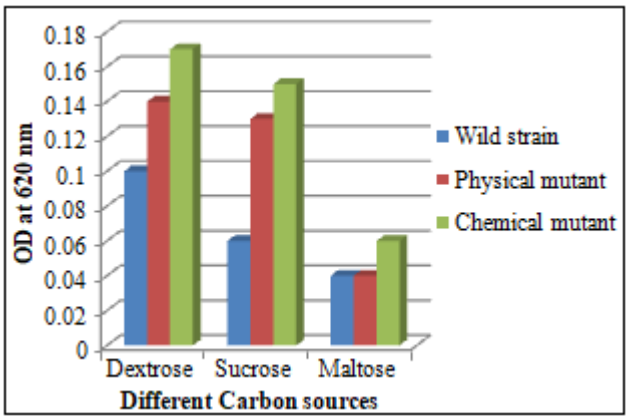

Figure 10: Dextrose was selected as best carbon source

3.7.4 Effects of nitrogen source

Table 5: Optimization of best nitrogen source

S no.	Culture	OD (620nm)		
		Peptone	Malt	NH_4Cl
1	Wild strain	0.04	0.16	0.02
2	Physical mutant	0.05	0.23	0.06
3	Chemical mutant	0.06	0.18	0.03

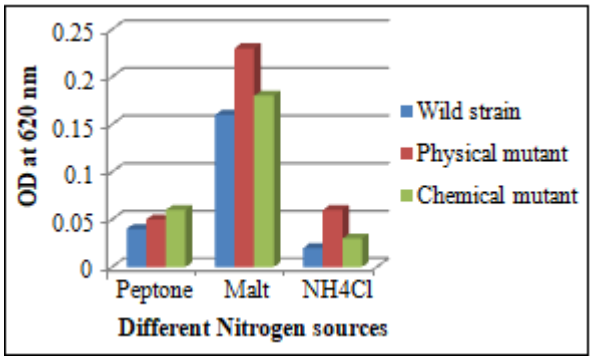

Figure 11: Media optimization of nitrogen source

3.7.5 Effects of temperature

Table 6: Different temperature was selected to optimize it

S no.	Culture	Remarks			
		4°C	Room temp.	37°C	50°C
1	Wild strain	-	+	++	+
2	Physical mutant	-	+	++	+
3	Chemical mutant	-	+	++	+

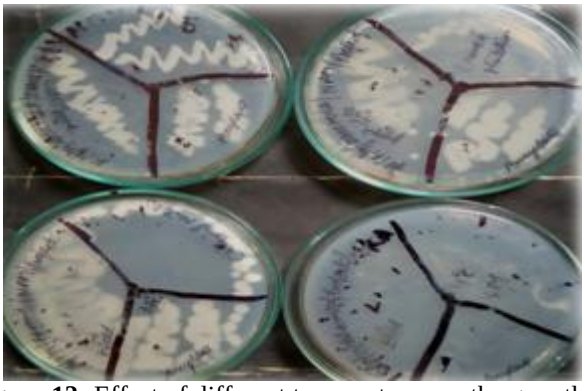

Figure 12: Effect of different temperatures on the growth of culture.

3.7.6 Effects of pH

Table 6: Different pH ranges were taken to optimize the pH for fermentation.

S no.	Culture	OD (620nm)			
		pH4	pH7	pH9	pH11
1	Wild strain	0	0.27	0.2	0.06
2	Physical mutant	0.01	0.28	0	0.08
3	Chemical mutant	0	0.26	0.1	0.07

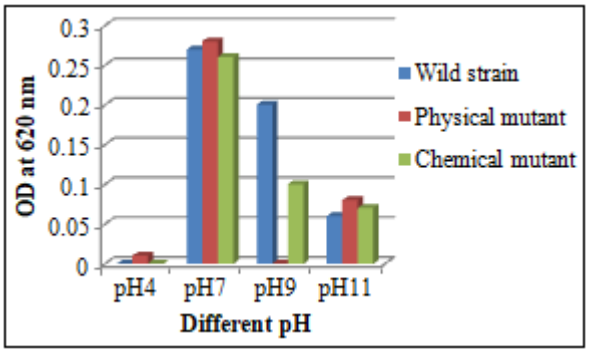

Figure 13: pH 7 was identified as best pH

3.8 Growth curve study of bacteria

Table 7: Bacterial growth curve study

S no.	Time in hr.	Wild Strain	Chemical mutant	Physical mutant
1	0	0.01	0.01	0.01
2	1	0.00	0.01	0.01
3	2	0.01	0.09	0.02
4	3	0.01	0.19	0.1
5	4	0.05	0.19	0.2
6	5	0.07	0.29	0.3
7	6	0.07	0.3	0.3
8	7	0.1	0.35	0.4
9	8	0.15	0.4	0.8
10	9	0.25	1	1.01
11	10	0.65	1.01	1.02
12	11	0.66	1.05	1.03
13	12	0.68	1.1	0.9
14	13	0.70	1.2	0.8
15	14	0.72	1.2	0.7
16	15	0.63	1	0.5
17	16	0.24	0.5	0.4
18	17	0.19	0.4	0.1

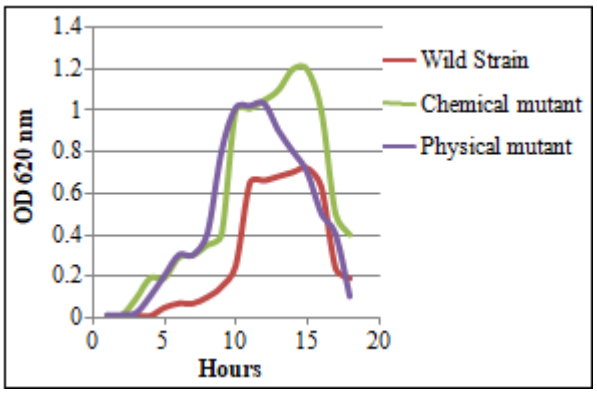

Figure 14: Bacteria growth curve

Fermentation and Downstream processing
Salt precipitation-
Purification of fermented enzyme was done by salt precipitation.

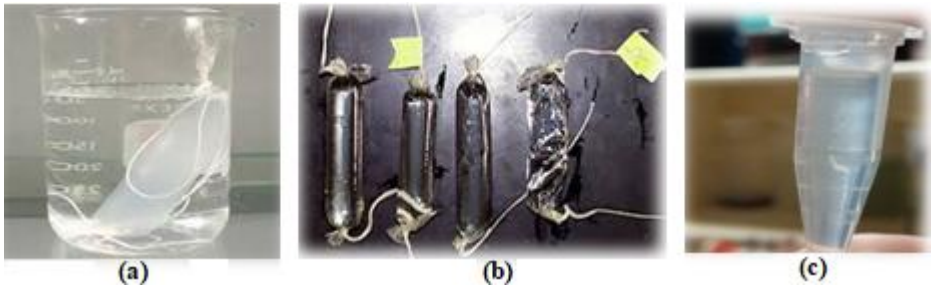

Figure 15: (a) dylasis bag in tris buffer, (b)Dylasis bags & (c) Enzyme

3.9 Enzyme assay

Table 8: Enzyme activity

Bacterial strain	OD (540 nm)	Activity units/ml/min
Wild strain	0.56	0.0029
Physical mutant strain	1.03	0.0060
Chemical mutant strain	1.46	0.0068

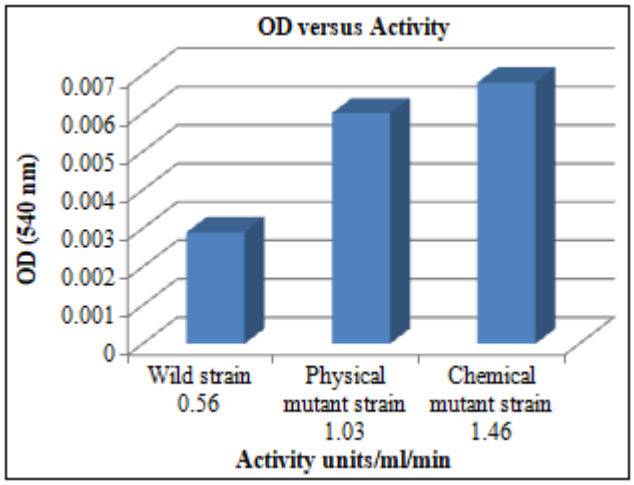

Figure 16: Comparative activity between wild and mutated strain

3.10 Extra property of physically mutated bacteria

There is one unique property of the bacteria which was observed. The wild strain is not able to produce the amylase. But after physical and chemical mutation it was observed that the mutation caused by UV treatment for 2 min. The extra property of the bacteria was observed for the amylase enzyme. The culture was screened for amylase by iodine flood test.

- For screening the amylase enzyme the minimal salt agar media was prepared and then sterilized.
- The culture was streaked and incubated for 48 hours, 37°C.
- Then the iodine flood was done and clear zone was observed in UV treated strains.
- The enzyme assay was also performed by using DNS test, for enzyme production test.

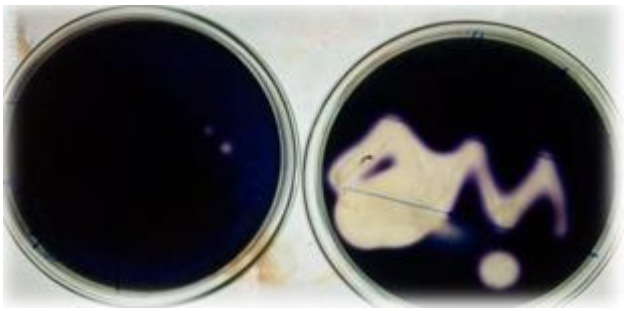

Figure 17: Screening of bacteria for amylase production by strach hydrolysis.

Sno	Bacterial strain	OD 540 nm	Activity units/ml/min
1	Blank	0	0
2	UV 2 min	1.36	0.0064

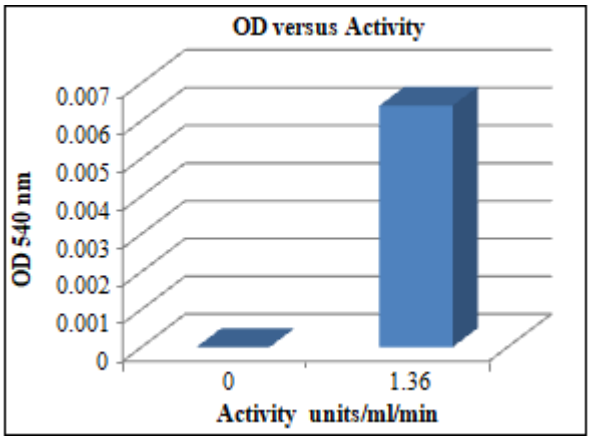

Figure 17: Amylase enzyme activity

4. Conclusion and Discussion

In this project ,we would first isolated the bacteria from straw then performed spreading, streaking process and then bacterial screening by the help of CMC agar media and Congo red solution ,and isolated desired bacterial strain is Bacillus Megaterium for strain identificationgram ‘straining test ,catalase test, Endospore test, Mannitad test, and vp tests were performed. For enhanced the production of cellulase enzyme, mutagen were used. For mutation physical and chemical mutagens were used.

For physical mutation, UV rays and chemical mutation Etbr were used. Media was optimized by one time one factor method for higher bacterial growth. These optimized media was used in fermentation for the production of cellulose enzyme. Thus we concluded that after mutation the production of cellulase enzyme in increased.

Mutated bacterial strain was streaked on starch plate and zone of hydrolyses was shown.

Thus finally we would conclude that after the mutation (both physical and chemical mutation) in wild strain of Bacillus megaterium the production of cellulase would increase and chemical mutation gave us best production of cellulose enzyme . After the physical mutation bacteria would contain both amylase and cellulase producing property and gave us best production of both amylases,cellulase enzyme.

5. Acknowledgement

I wish to express my immense gratitude to Mr. Manoj Verma, Director, MRD LifeSciences Pvt. Ltd. Lucknow. I am very grateful and my heartiest thanks to Ms. Shraddha Prakash (Research Scientist), Chitranshu Pandey (Research Scientist) and Pragya Srivastava (Research Assistant) MRDLS, Lucknow, for there kind support throughout the research work, I am also thankful to the almighty without whose blessings nothing was possible.

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