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Isolation, Screening and Optimization of Microbial Cellulase

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Abstract: **Cellulase enzymes are biological molecules that act as a catalyst and help complex reaction to occur. Now day significant attentions have been devoted to the current knowledge of cellulase production and challenges in cellulase research especially in the direction of improving the process economics of various industries.Having potential application in various industries including pulp and paper, textile, food and laundry industries. Enzyme cellulase cause hydrolysis of the individual cellulose fibers to break it into smaller sugars units and finally producing glucose molecules. The cellulase producing bacteria were isolated from paper industry waste. A total of 9 isolates were obtained by the qualitative screening technique. Isolates coded PP-1 and PP-2 were efficient cellulase producers with specific enzyme activity of 181mU/ml and 189mU/ml respectively.Organism coded PP-2 gives maximum enzyme activity when characterized for pH, temperature, carbon and nitrogen sources.As pH 7.0,temperature 37˚C, fructose andammonium phosphate as best carbon and nitrogen source. PP-2 counts to be best cellulase producer among the rest 9 isolates, having optimum growth condition between pH 5-7 and temperature 30˚C – 40˚C. Potent isolate was characterized on the basis of morphological, structural, biochemical test and partially identified as Bacillus Sp. with the help of Bergey'sManual of systematic bacteriology.**

***Keywords*: Cellulose, cellulase, paper pulp industries**
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I. INTRODUCTION

Cellulose is the most common organic polymer, representing about 1.5 x 1012 tons of the total annual biomass production from various sources [1]. Microbial degradation of cellulosic waste and the downstream products resulting from it is accomplished by a concerted action of several enzymes, the most prominent of which are the cellulases, which are produced by a number of microorganisms and comprise several different enzyme classifications. Cellulases hydrolyze cellulose (β-1, 4-D-glucan linkages) and produce as primary products glucose, cellobiose and cello-oligosaccharides.

There are three major types of cellulase enzymes cellobiohydrolase (1,4-β-D-glucancellobiohydrolase, EC 3.2.1.91), Endo-β-1,4-glucanase (endo-1,4-β-D-glucan 4-glucanohydrolase, EC 3.2.14) and β-glucosidase (BG-EC 3.2.1.21)] [1]. Enzymes within these classifications can be separated into individual components, such as microbial cellulase and non-microbial.

Cellulolytic microbes are primarily carbohydrate degraders and are generally unable to use proteins / lipids as energy sources for growth[2]. Bacteria and most fungi can utilize a variety of other carbohydrates in addition to cellulose[3 and 4], while the anaerobic celluloytic species have a restricted carbohydrate range, limited to cellulose and or its hydrolytic products[5 and 6] The ability to secrete large amounts of extracellular protein is characteristic of certain fungi and such strains are most suited for production of higher levels of extracellular cellulases. One of the most extensively studied fungi is Trichodermareesei, which converts native as well asderived cellulose to glucose.

Now a day significant attentions have been devoted to the current knowledge of cellulase production and challenges in cellulase research especially in the direction of improving the process economics of various industries.

Cellulases are used in the textile [7 and 8], in detergents[9], pulp and paper industries[10], also for improving digestibility of animal feeds [11] and the enzymes also account for a significant share of the world enzyme market.

II. METHOD

A.Sample Collection and Isolation of cellulolytic bacteria

Soil sample was collected for isolation of cellulolytic bacteria from different region of south Gujarat. Cellulolytic bacterial strains were isolated from soil by using serial dilutions and pour plate technique. The medium used for isolation of cellulolytic bacteria contains;

Medium Composition (for 1 Liter) :

Carboxymethylcellulose Agar:

NH_4H_2PO4 -1.0 gm, KCl-0.2 gm, $MgSO_4.7H_2O$ - 1.0 gm, Yeast extract - 1.0 gm, Carboxymethylcellulose - 20.0 gm, Agar agar powder -30.0 gm.

Bacterial colonies were purified by repeated streaking. The purified colonies were preserved at 4˚C for further identification and screening for cellulase production.

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B.Primary screening of cellulolytic bacteria:

The incubated CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature. 1M NaCl was thoroughly used for counterstaining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The bacterial colonies having clear zone were selected for identification and cellulase production. Further bacterial strains were purified by repeated streaking. The purified colonies were preserved at 4 ˚C. [12]

C. ***Identification of Isolate:***

Cultural characterization

The isolates were observed under microscope. The colony morphology was noted with respect to color, shape, size, nature of colony and pigmentation. [12]

Microscopic observation

The bacterial isolation was gram stained and observed under a high power magnifying lens in light microscope.

Biochemical characterization

The bacterial isolates were characterized biochemically by various test.

D.Inoculums Development:

The selected bacterial cultures were individually maintained on nutrient agar slants at 4 ˚C. The selected bacterial cultures were inoculated in broth medium containing 1 % peptone, 0.5 % NaCl and 0.3% meat extract at pH 7 for 24 Hrs of incubation period. After the incubation period these bacterial cells were used as inoculum.

E. **Cellulase enzyme assay:**

Carboxymethylcellulase(CMCase) activity was estimated using a 1 % solution of carboxymethlycellulose (CMC) in 0.05 M citrate buffer (pH 4.8) as substrate. The reaction mixture contained 1 ml citrate buffer, 0.5 ml of substrate solution and 1ml of crude enzyme solution. The reaction was carried out at 45 ˚C for 30 min. The amount of reducing sugar released in the hydrolysis was measured by DNSA method. The Enzyme unit (EU) was determine as the amount of CMCase required to release 1µmole of reducing sugar per ml per minute under above assay condition.[13]

F.Optimization of Cellulase Enzyme:

Effect of different pH on cellulase production

Flasks with broth containing the optimum concentration of substrate and carbon source are taken and the pH of the broth is adjusted to 7.0, 8.0, 9.0, 10.0, and 11.0 in different flasks using 1NHCl and 1NNaOH and sterilized. The cultures are inoculated and incubated at particular temperature. At the end of incubation period, the cell-free culture filtrate is obtained and used as enzyme source.

Effect of different temperature on cellulase production

Production medium at pH 7 was inoculated with overnight grown selected bacterial strain. The broth was incubated at different temperatures from 37, 4 and 50˚C for 24 hours. At the end of incubation period, the cell-free culture filtrate is obtained and used as enzyme source.

Effect of different carbon sources on cellulase production

The effect of various carbon sources such as starch, cellulose powder, glucose, maltose, sucrose, fructose, groundnut shell, rice straw, rice bran, and wheat bran at the concentration of 1% was examined in the production medium.

Effect of different nitrogen sources on cellulase production

Various nitrogen sources like meat extract, peptone, urea, ammonium chloride, ammonium phosphate, beaf extract, ammonium nitrate, gelatinand ammonium sulphate were examined for their effect on enzyme production by replacing 0.5% yeast extract in the production medium.

III. RESULT AND DISCUSSION

A. ***A.Isolation and screening for cellulase producing bacteria***

Total 5 samples were collected from 5 different sites and total 9 isolates were obtained. From these, 1 out of 9 isolates were further studied due to positive isolates. The resulting 5 isolates were then tested on CMC agar for cellulase activity. Their CMCase activity is shown in Table 2.

For secondary screening the bacterial colonies were spotted on fresh CMC agar media incubated at 37 ˚C for 48 hour. The cellulase producing bacterial strain were identified by the zone of clearance after stain the plate with 1% Congo-red solution for 15 min and counterstain the plate with 1M NaCl for 15 min.

Table 1: Colony characteristics, motility and Gram Staining

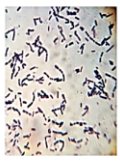
Sample No.	Name of the medium	Isolate No.	Motility	Gram Reaction and Morphology	Colony Characteristics		Figure
1	Nutrient agar medium	PP2	Motile	Gram positive short rods occurring singly and in chain	Size	Small	
					Shape	Round	
					Edge	Entire	
					Elevation	Convex	
					Texture	Smooth	
					Consistency	Moist	
					Opacity	Opaque	
					Pigmentation	No	

Table 2: Growth characteristics on CMC agar medium

Sample No.	Name of the medium	Isolate No.	Colony/ Growth Characteristics	Figure
1	Carboxymethyl cellulose agar medium	PP2	Small, Round, Entire, Smooth, Moist, Opaque	

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Table 3: Biochemical Test for Isolate PP2

Name of the Biochemical test	Result
Indole test	-
MR test	-
VP test	-
Citrate utilization test	-
H_2S production test	-
Nitrate reduction test	-
Urea hydrolysis test	-
Gelatin liquefaction test	+
Sugar fermentation test	
Nutrient glucose broth	A
Nutrient sucrose broth	-
Nutrient xylose broth	A
Nutrient mannitolbrth	A
Nutrient mannose broth	A
Nutrient maltose broth	-
TSI agar slant	
Slant	Yellow
Butt	Yellow
CO_2 Production	-
H_2S Production	-
Enzyme profile	
Casein	+
Amylase	-
Gelatinase	+
Catalase	+
Oxidase	-

(**Legends**: A= Acid, Al= Alkaline, G= Gas, += Positive test, -= Negative test)

B. Estimation of Cellulase enzymeEstimation of reducing sugar by DNSA method

Cellulase activity (CMCase) was determined by estimating the librated amount of reducing sugar using DNS method as per Miller (1995). Glucose was used as the standard in this assay. Culture supernatant was used for estimation of reducing sugar.

Filter paper assay

The FPA of the original concentrated enzyme solution was calculated from above formula. One filter paper unit (FPU) was defined as the amount of enzyme releasing 1 mole of reducing sugar from filter paper /ml /h.The calculation of CMCase and FPase activity is by given formula [14],

$$\text{Enzyme activity} = \frac{\text{Amount of product formed}}{\text{Molecular weight of CMC} \times \text{Vol. of enzyme (ml)} \times \text{Incubation time (mins.)}}$$

Note: Molecular weight of CMC is 263.19 g/mole.

Protein Estimation

A. Protein content of the isolated strain was estimated by using the Folin Lowry's method and optical density of the strain was compared with the BSA standard curve to calculate the amount of protein (mg/ml) present in the supernatant used in cellulase assay.

Table 4: Specific activity of isolate PP2

Bacterial strain	CMCase activity (U/ml)	FPase activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg) by CMCase activity	Specific activity (U/mg) by FPase activity
PP2	0.287	0.03	0.225	1.27	0.13

C. C.Effect of various parameters on cellulase enzyme production

Effect of pH on Cellulase Enzyme Production

The optimum pH for cellulase enzyme production was studied by preparing the production medium with pH values ranging from 3.0,3.2,3.4,3.6 to 10.0. The flasks were inoculated with selected microbial strain and incubated in rotatory shaker incubator. After incubation, the culture filtrates were assayed for cellulase production according to DNSA method. The following figure shows the effect of pH on enzyme production.

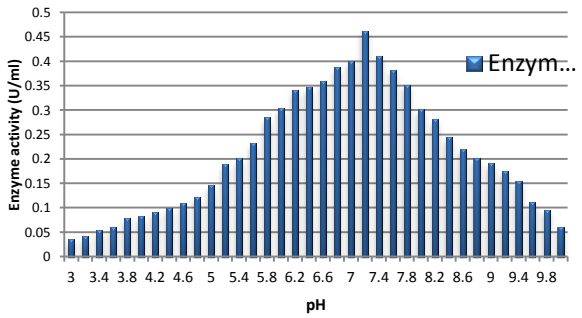

Figure: Effect of pH on cellulase enzyme production

D. The results of the above graph clearly showed that as the ***pH*** increases, cellulase enzyme activity also increased till pH 7.2, remained stationary till pH 7.4 and then declined. The optimum pH was found to be 7.2 where cellulase activity was maximum. Our results were in correspondence with the work of Balamurugan et al., (2011). Thus, the optimum pH was found to be 7.2 at which cellulase enzyme activity was maximum(0.462 U/ml).

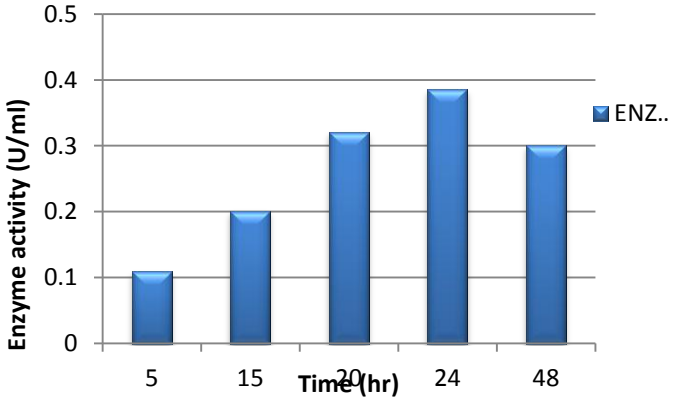

Figure: Optimum pH 7.2 on different incubation period

Effect of Temperature on cellulase enzyme production

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The temperature optimum was studied by incubating the production medium (pH 7.2) inoculated with 1% inoculum at varying incubation temperature such as 4,10,20,37,40,50,60,80 ˚C for 48 hour at 100 rpm. The culture filtrates were retrieved and assayed for cellulase production.

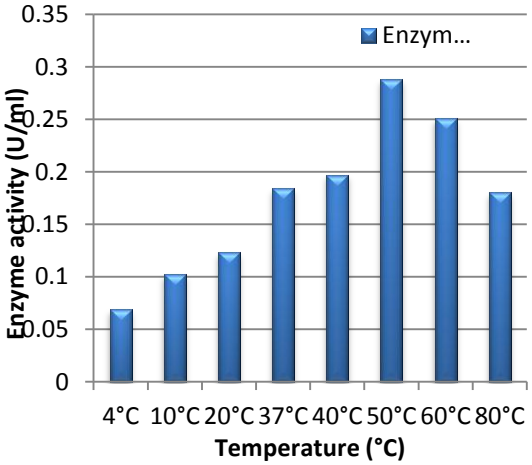


Figure: Effect of temperature on cellulase enzyme production

From the above Figure, it was found that with increase in temperature range from 10 – 30 ºC, cellulase enzyme activity also increased. There was no further increase in enzyme activity with increase in temperature ranging from 60 – 80 ºC. The optimum temperature was found to be 50 ºC at which maximum enzyme activity (0.288 U/ml) was achieved. Thus, 50 ºC was optimum temperature.

The increase with temperature is due to corresponding increase in kinetic energy and the decline after the optimum due enzyme denaturation (Zakpaa*et al*., 2009; Sherief*et al.*, 2010; Gautam*et al*., 2011).

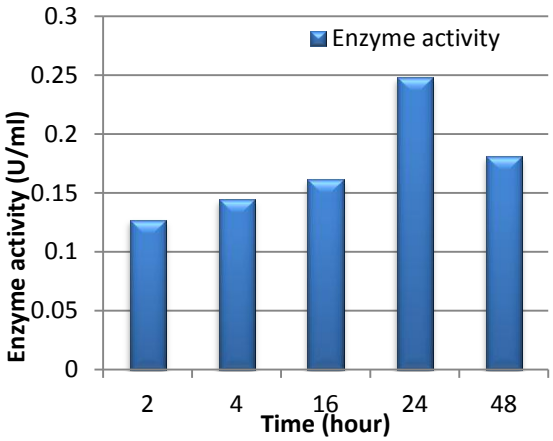


Figure: Effect of incubation time at 50°C

Effect of inoculum concentration on cellulase enzyme production

In order to determine the influence of inoculum concentration on cellulase enzyme production, various concentrations of inoculum were used (0.1 – 5.0%). Culture filtrate retrieved after incubation was estimated to determine the cellulase enzyme activity according to DNSA method.

It is clear from the below figure that cellulase activity increases as the inoculum concentration increases. The optimum inoculum concentration was found to be 2% at which maximum cellulase activity of 0.284 U/ml was achieved.

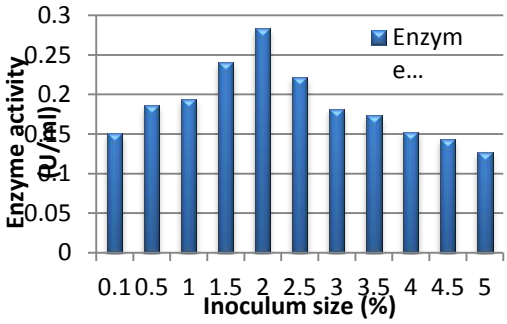
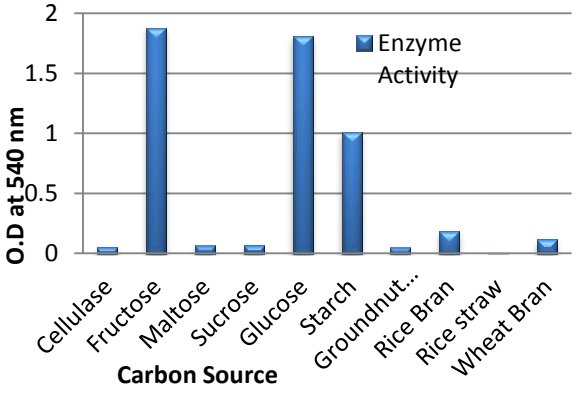


Figure: Effect of inoculum concentration on cellulase enzyme production

Effect of carbon supplementation on cellulase enzyme production

The effect of carbon supplementation to the culture broth on cellulase enzyme production was performed by inoculating the selected carbon sources such as glucose, starch, sucrose, fructose and maltose at 1.0% concentration. The inoculated culture flasks were incubated at optimized conditions and glucose released was calculated.

Figure: Optimization of Car



bon supplementation for maximum cellulase activity

From above figure, it was found that among the different carbon supplements used for enhanced cellulase production,

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the culture flasks supplemented with 1.0% concentration of fructose and glucose showed maximum cellulase activity (1.878 U/ml and 1.81 U/ml respectively).

Effect of nitrogen supplementation on cellulase enzyme production

Similar to the carbon supplementation studies, effect of nitrogen supplementation to the culture broth was studied and the optimum concentration of the selected source was standardized. Under optimized culture conditions, the inoculated culture flasks were incubated and the amount of glucose released was estimated to determine cellulase activity.

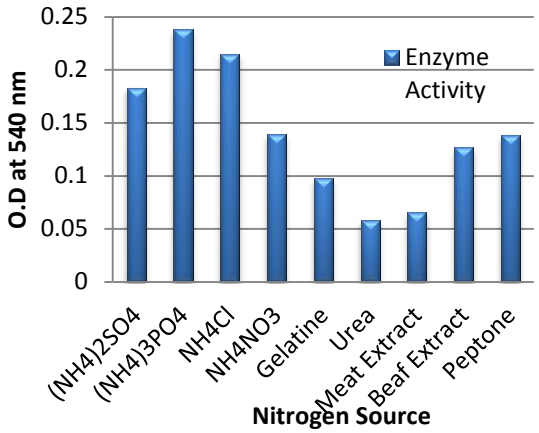

Figure: Optimization of Nitrogen supplementation for maximum cellulase activity

The above figure clearly showed that supplementation of nitrogen source to the cellulase production medium had profound influence on enzyme activity. It was found that ammonium phosphate supplementation showed maximum enzyme activity of 0.238 U/ml at 1.0% concentration.

IV.CONCLUSION

Cellulosic polymeric wastes are now day's major pollutant to the environment. During the course of research, bacterial isolates were obtained degrading cellulose. Potent cellulase producing isolate PP2 was partially identified as *Bacillus* sp. on the basis of morphological and biochemical characteristics with the help of *Bergy's Manual* of systemic bacteriology. Crude enzyme production sufficiently increased with use of fructose and ammonium phosphate as carbon and nitrogen sources respectively. Cellulase found to be active at different pH and temperature ranges. Variation in size of inoculum also found to affect enzyme production. Considering these all aspects crude cellulase obtained from PP2 can be further used for environmental and commercial purposes.

REFERENCES

1. Sukumaran, R. K., Singhania, R. R., and Pandey, A. (2005).Microbial cellulases-production, applications and challenges. Journal of Scientific and Industrial Research, 64(11), 832.

2. Lynd, L. R., Weimer, P. J., Van Zyl, W. H., and Pretorius, I. S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. Microbiology and molecular biology reviews, 66(3), 506-577.

3. Poulsen, O. M., and Petersen, L. W. (1988).Growth of Cellulomonas sp. ATCC 21399 on different polysaccharides as sole carbon source induction of extracellular enzymes.Applied microbiology and biotechnology, 29(5), 480-484.

4. Rajoka, M. I., and Malik, K. A. (1997).Cellulase production by Cellulomonasbiazotea cultured in media containing different cellulosic substrates.Bioresource Technology, 59(1), 21-27.

5. Ng, T. K., and Zeikus, J. G. (1982).Differential metabolism of cellobiose and glucose by Clostridium thermocellum and Clostridium thermohydrosulfuricum. Journal of Bacteriology, 150(3), 1391-1399.

6. Thurston, B., Dawson, K. A., and Strobel, H. J. (1993).Cellobiose versus glucose utilization by the ruminal bacterium Ruminococcusalbus.Applied and environmental microbiology, 59(8), 2631-2637.

7. Gusakov, A. V., Berlin, A. G., Popova, N. N., Okunev, O. N., Sinitsyna, O. A., and Sinitsyn, A. P. (2000). A comparative study of different cellulase preparations in the enzymatic treatment of cotton fabrics.Applied biochemistry and biotechnology, 88(1-3), 119-126.

8. Belghith, H., Ellouz-Chaabouni, S., and Gargouri, A. (2001). Biostoning of denims by Penicilliumoccitanis (Pol6) cellulases. Journal of Biotechnology,89(2), 257-262.

9. Maurer, K. H. (1997). Development of new cellulases.Surfactant science series, 69, 175-202.

10. Buchert, J., Suurnakki, A., Tenkanen, M., and Viikari, L. (1996).Enzymatic characterization of pulps.

11. Lewis, G. E., Hunt, C. W., Sanchez, W. K., Treacher, R., Pritchard, G. T., and Feng, P. (1996). Effect of

direct-fed fibrolytic enzymes on the digestive characteristics of a forage-based diet fed to beef steers. Journal of Animal Science, 74(12), 3020-3028.

12. Tomme, P., Warren, R. A. J., and Gilkes, N. R. (1995).Cellulose hydrolysis by bacteria and fungi.Advances in microbial physiology, 37(1), 1-81.

13. Shaikh, N. M., Patel, A. A., Mehta, S. A., and Patel, N. D. (2013). Open Access Research Article.

14. Segneanu, A. E., Cziple, F., Vlazan, P., Sfirloaga, P., Grozescu, I., and Gherman, V. D. (2013).Biomass extraction methods. In Biomass Now-Sustainable Growth and Use Intech open science, p 540

15. Bergy's manualof systematic bacteriology.