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RESEARCH ARTICLE

ISOLATION, SCREENING, PRODUCTION, MEDIA OPTIMIZATION, PARTIAL PURIFICATION AND CHARACTERISATION OF CELLULASE PRODUCING *BACILLUS SPECIES* ISOLATED FROM RHIZOSPHERE SOIL

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ABSTRACT

The cellulase producing *Bacillus species* was isolated from rhizosphere soil and characterized by various staining procedures, biochemical analysis and partial purification. Further partial purification of cellulase was carried out by dialysis and ammonium phosphate precipitation and also to determine molecular weight by SDS-PAGE. Bacterial isolated were grown on Carboxy Methyl Cellulose (CMC) agar at various optimum conditions such as parameters like pH, temperature, incubation period, carbon and nitrogen sources prior to examined and identified primarily as genus, Bacillus species for cellulase production by morphological and biochemical analysis. The cellular enzyme was completely active in a large pH range (5 -8) and presented an optimum activity of 195U/mL. The cellulase production was found to be more when incubated for two days (48 hrs). Ammonium sulphate precipitation followed by dialysis was performed to partially purify the cellulase enzyme. The molecular weight was found to be 32 kilodaltons by SDS-PAGE method. The specific activity of the enzyme was found to be 0.3 mg/mL. The use of microorganisms for the production of enzymes offers a promising approach for its large scale production and as a possible food supplement or in pharmaceutical industry.

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INTRODUCTION

Bacillus sp. is the most commonly used microorganism for the industrial production of enzyme cellulase. Cellulases have a wide range of industrial applications such as textile, laundry, pulp and paper, fruit juice extraction, and animal feed additives as well as in bioethanol production. This bacterium occurs in rich natural environment and commonly in soil. *Bacillus* sp. can be isolated from different sources water, air and fermented foods. Among this soil is the best the source for bacilli. Also, soil caters the growth of bacilli like, PH, temperature, nutrient and moisture.Cellulose is the most abundant biomass on the earth. Cellulases are inducible enzymes which are synthesized by large number of microorganisms either cell-bound or extracellular during their growth on cellulosic materials (Lee andKoo, 2001). Cellulose, acrystalline polymer of D-glucose residues connected by β-1, 4 glucosidic linkages, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature (Saha*et al*., 2006).

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The cellulases have great potential in saccharification of lignocellulosics to fermentable sugars which can be used for production of bioethanol, lactic acid, and single cell protein. Therefore, it has become considerable economic interest to develop processes for effective treatmentand utilization of cellulosic wastes as inexpensive carbon sources. Complete enzymatic hydrolysis of enzyme requires synergistic action of 3 types of enzymes, namely cellobiohydrolase, endoglucanase or carboxy methyl cellulose (CMCase) and α-glucosidases (Bhat, 2000). Cellulose is commonly degraded by an enzyme called cellulase. Cellulase refers to a class of enzymes produced chiefly by fungi, bacteria, and protozoans that catalyse the cellulolysis (or hydrolysis) of cellulose (Immanuel *et al*., 2006). This enzyme is produced by several microorganisms, commonly by bacteria and fungi. Cost of cellulase in enzymatic hydrolysis is regarded as a major factor (Kanokphorn *et al*., 2011). Cellulases have attracted much interest because of the diversity of their application. The major industrial applications of cellulases are in textile industry for 'bio-polishing' of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness (Cavaco-Paulo, 1998).Application of enzymes in textile, food, detergent, leather and paper industries demands

identification of highly stable enzymes active at extreme pH and temperature (Abdelnasser and Ahmed, 2007). Cellulase is used in the fermentation of biomass into biofuels (Cherry and Fidantsef, 2003), fibre modification and they are even used for pharmaceutical applications. Bacteria has high growth rate as compared to fungi has good potential to be used in cellulase production. Celluloytic property of some bacterial genera such as Cellulomonas species, Pseudomonas species, Bacillus species and Micrococcus species were reported (Nakamura and Kappamura, 1982). Enzyme production is closely controlled in microorganisms and for improving its productivity these controls can be ameliorated. Cellulase yields appear to depend on a complex relationship involving a variety of factors like inoculum size, pH, temperature, presence of inducers, medium additives, aeration, and growth time (Robson and Chambliss, 1989). The aim of this study was to isolate and screening of cellulase producing bacteria from cow dung, optimization of conditions, production of cellulase, partial purification determination of Molecular weight.

MATERIALS AND METHODS

Isolation of cellulase producing *Bacillus* sp. from soil

Bacillus sp. is the most commonly used microorganism for the industrial production of enzyme cellulase. This bacterium occurs in rich natural environment and commonly in soil. *Bacillus* sp. can be isolated from different sources water, air and fermented foods. Among this soil is the best the source for bacilli. Also, soil caters the growth of bacilli like, PH, temperature, nutrient and moisture.

Purification of bacteria

Microorganisms in nature occur only as mixed cultures. A culture that is grown from a single cell is called as pure culture. The pure cultures are necessary to study the colony characteristics and immunological reaction of particular bacteria, fungi or actinomycetes.

Three methods *viz*.

- Streak plate method
- Pour plate method
- Spread plate method

Are commonly employed for obtaining pure cultures of bacteria. The general principle involved in this technique is to dilute or divided the mixed population so that the single cells are separated and develop in to single colonies. The individual colonies are picked up and grown as pure culture i.e. growth derived from a single cell.

Isolation procedure

A moisture part of the soil from garden was selected. The debris from the top soil was removed and a sample of soil was collected in a Ziploc bag.

Serial dilution and standard plate counts

This technique relies on the fact that under proper conditions. Only living bacteria will divide and form a visible colony on an agar plate. An agar plate Petridish containing a nutrient medium solidified with agar. Since the count is more than 300colonies on one agar plate, is necessary dilute the original bacterial culture before we transfer a known volume of the culture before on to the solid plate, serial dilutions accomplish this purpose. The first step in making a serial dilution is to take a known volume (usually 1mL) of stock and place it into a known volume of distilled water (usually 9 mL). This produces 10mL of dilute solution. The dilute solution has 1 mL of extract /10 mL. This is a fold dilution. The concentration of stock in each mL of diluted solution is 1 mL. The technique used to make a single dilution is repeated sequentially using a more and more dilute solution as the "stock'' solution. At each step 1mL of the previous dilution is added to 9mL of distilled water. Each step result in a 10 fold change in the concentration from the previous concentration. The values shown in the tubes are the amount of stock present in each mL of dilution solution. By using pour plate technique the dilution samples were plated in nutrient agar medium and incubated at 37^0C for overnight incubation. The isolated colonies observed for following colony characteristics.

Pour plating method

Melt three tubes of nutrient agar medium (12 - 15 mL) and cool them to 45^0C. Transfer one gram of the soil sample in first tube of sterile water blank. Mix the soil sample well by thumbing the tube with fore finger. (The success of the method depends upon through mixing and even distributed of the cells).Transfer 1 mL of soil sample from the first tube to a second tube of sterile water blank and mix the contents well as stated above. Transfer similarly from the second to third tube of agar and mix thoroughly. Pour each of the tubes in to separate Petri plates by rotating clockwise and anticlockwise direction to have uniform distribution. Incubate the Petri plates at bacterial incubator for 24 h depending on the organism incubation period will change.

Gram's staining

Gram's staining is the single most useful test in the microbiology laboratory given its simplicity and ability to differentiate bacteria two main groups: gram - positive organisms and gram negative organisms. Make a thin smear of the bacterial suspension on a grease free slide. Stain the smear with ammonium oxalate crystal violet for a minute. Wash in tap water for 10 second. Flood the smear with Lugol's iodine solution for 1 minute. Wash in tap water for 10 second and blot dry. Decolorize with 95% ethanol for 30 second. Counter stain with Safranin solution for a minute. Wash gently in tap water. Blot dry and examine under oil immersion (100x) objective.

Screening for cellulose production

Isolates were inoculated on cellulose agar medium in plates and they were incubated for 2 days. The plates were added with 3 mL congo red and allow it for 30 minutes. 3 mL of NaCl was added to the plates and allowed it for 20 minutes, clear zone was formed on the plates.

Estimation of enzyme activity

After incubation DNS Assay (Dinitrosalicyclic Acid) was performed to determine the enzyme activity. Cultures were

filtered with normal filter paper and centrifuged at 5000 rpm for 20 minutes at 4˚C. 0.5 mL of supernatant (enzyme extract) was taken in a fresh test tube and 1mL of 1% CMC in Phosphate buffer (Substrate) was added and kept for incubation at 37˚C for 30 minutes. 3 mL of Dinitrosalicyclic Acid (DNS) reagent was added and the tubes were boiled in water bath for 15 minutes. 1 mL of Rochelle salt solution (40% Potassium Sodium Tartarate) was added to the tubes after it was cooled. 1 mL CMC in Phosphate buffer and 0.5 mL distilled water was used as blank. The colour intensity was determined by Spectrophotometric analysis at 540 nm.

Media optimization

Media optimization was done to standardize the conditions favouringcellulase growth, to get high yield of the enzyme. Modification was carried out with different levels of carbon sources, nitrogen sources, pH, temperature, percentage of inoculum size, incubation period. Enzyme activity was determined by DNS Assay.

Optimization parameters

Effect of various carbon sources

Ten different carbon sources were used as substrates for the cellulase production, namely Carboxy Methyl Cellulose (CMC), Cellulose powder, filter paper, normal paper. To four culture tubes, 15 mL of carbon substrates broth is distributed. 1% of different carbon sources were added. The bottles were labelled according to the carbon source. pH of the medium was adjusted to 7.2 and autoclaved at 121˚C for 20 minutes. After cooling of the medium all bottles were inoculated with 0.15 mL (1% v/v) of the test culture. Following inoculation the bottles were incubated at 30˚C in shaker incubator for 2 days. Following incubation, the broths were filtered using watman filter paper. Using the filtrates as crude enzyme extracts and Carboxy Methyl Cellulose as the substrate, the enzyme assay was carried out using DinitroSalicyclic acid (DNS) method.

Effect of various nitrogen sources

Ten different nitrogen sources were used as substrates for the cellulase production, namely Peptone, Tryptone, Yeast extract, Beef extract, Ammonium Chloride (NH4Cl), Ammonium Nitrate (NH_4NO_3), Sodium Nitrate ($NaNO_3$) and Sodium Nitrite ($NaNO_2$).To eight culture tubes, 15 mL of CMC broth was distributed. 1% of different nitrogen sources were added. The bottles were labelled according to the nitrogen sources. pH of the medium was adjusted to 7.2 and autoclaved at 121˚C for 20 minutes. After cooling of the medium all bottles were inoculated with 0.15 mL (1% v/v) of the test culture. Following inoculation the bottles were incubated at 30˚C in shaker incubator for 2 days and the cellulase activity was determined by DNS method.

Effect of different pH

A pH range of 4 – 9 was selected for studying the effect of various pH on the growth of cellulase. To six culture tubes, 15 mL of CMC broth was distributed. 1% of carbon source and 0.1% of nitrogen sources were added. The bottles were labeled according to the pH range. pH of the medium was adjusted from 4 to 9 and autoclaved at 121˚C for 20 minutes. After cooling of the medium all bottles were inoculated with 0.15 mL (1% v/v) of the test culture. Following inoculation the bottles were incubated at 30˚C in shaker incubator for 2 days and the cellulase activity was determined by DNS method.

Effect of different temperature

Three temperatures were selected for this study (25˚C, 30˚C and 37˚C). To three culture tubes, 15 mL of CMC broth was distributed. 1% of carbon source was added. The bottles were labelled according to the temperature range. pH of the medium was adjusted to 5 and autoclaved at 121˚C for 20 minutes. After cooling of the medium all bottles were inoculated with 0.15 mL (1% v/v) of the test culture. Following inoculation bottles were incubated in 25˚C, 30˚C and 37˚C shaker incubator for 2 days and the cellulase activity was determined by DNS method.

Effect of different concentrations of inoculums size

To six culture tubes, 15 mL of CMC broth was distributed. 1% of carbon source was added. pH of the medium was adjusted to 5. The bottles were labelled according to the different concentrations of inoculums and autoclaved at 121˚C for 20 minutes. After cooling of the medium all bottles were inoculated with 0.25%,0.5%, 1%, 3%, 5% and 10% of the test culture respectively. Following inoculation the bottles were incubated at 30˚C in shaker incubator for 5 days and the cellulase activity was determined by DNS method.

Effect of different incubation period

Seven culture tubes containing 15 mL of CMC broth each was taken with 1% carbon source. pH of each bottle was adjusted to 5. The tubes were labeled according to the different incubation period and autoclaved at 121˚C for 20 minutes. After cooling of the medium all bottles were inoculated with 0.15 mL (1% v/v) of the test culture. Following inoculation the bottles were incubated at 25˚C in shaker incubator for 7 days and the cellulase activity was determined by DNS method every day till incubation period completes.

Mass production of cellulose

On the basis of the results obtained with all the optimized parameters mass production of the enzyme was carried out with 250 mL of optimized media. It was inoculated with the test culture and kept in a shaker incubator at 30˚C at 150 rpm for 5 days. A small amount of broth was filtered and centrifuged at 8000 rpm for 20 mins at 4°C and the DNS assay was performed to obtain enzyme activity. The remaining broth was centrifuged at 5000 rpm for 30 mins at 4°C and the supernatant was collected and subjected to protein estimation and purification.

Partial purification of enzyme

Ammonium sulphate precipitation

The crude sample was collected in a sterile chilled centrifuge tube and an equal volume of saturated ammonium sulphate solution was added to it. The contents were mixed thoroughly

and kept for precipitation, at -20˚C, overnight. The mixed contents were centrifuged at 5000 rpm for 30 minutes at 4˚C. The pellet was collected and suspended in phosphate buffer (pH 6.0) and stored at 4˚C. Then DNS assay was performed to determine the enzyme activity.

Dialysis

Dialysis tubing of suitable diameter was selected and cut to a suitable length, enough to contain about 1.5 mL volume. The tube was boiled for 10 minutes in 2% sodium carbonate solution. The solution was discarded and the tube was boiled in distilled water for 10 minutes thrice. The tube was then boiled in 0.05% EDTA for 10 minutes. One end of the dialysis bag was tied with rubber band and the sample was added into the tube and the other end was also tied. The tube was placed in a beaker containing distilled water. The contents of the beaker were then agitated gently with magnetic stirrer under refrigerated condition. It was stirred for 24 hours, allowing it to reach equilibrium. The purified protein was collected and stored at 4˚C and then with DNS assay enzyme activity was determined.

Quantification of protein by Lowry's method

Protein content was determined by the Lowry's method with crystalline Bovine Serum Albumin (BSA) as the standard (Lowry *et al*., 1951). For protein estimation, the working standard was pipetted out as 0.2, 0.4, 0.6, 0.8, and 1 mL of BSA into a series of test tubes. 0.2 mL of the crude enzyme sample extract, precipitated enzyme sample and dialysed enzyme sample was pipetted out into three test tube. The volume was made up to 2 mL in all test tubes, with distilled water. A tube of 2 mL distilled water instead of the enzyme extract or working standard served as the blank. 5 mL of Reagent C was added to all tubes including the blank. The contents of the tubes were mixed and allowed to stand for 10 minutes at room temperature. Then 0.5 mL of Reagent D (Folins – Ciocalteau Reagent) was added, mixed well and incubated at room temperature in the dark condition for 20 minutes. The colour intensity was read at 670 nm to estimate the amount of total protein present.

Molecular weight determination by SDS page

Electrophoresis is the study of the movement of charged molecules in an electric field. The generally used support medium is cellulose or thin gels made up of either polyacrylamide agarose. Cellulose is used as support medium for low molecular weight biochemical such as amino acid and carbohydrates whereas agarose and polyacrylamide gels are widely used for larger molecules like proteins. The glass plate was cleaned with methanol to make it grease free, and clamped for making leak proof, then the freshly prepared resolving gel was added and was allowed to polymerize. The top of gel was washed with distilled water and wiped with filter paper. Later on stalking gel was poured over the resolving gel and comb was inserted and was allowed to polymerize for 1 hour. Meanwhile, the samples (25 µL sample + 15 µL gel loading buffer) were denatured at 95˚C for 5 minutes, after polymerization the comb was removed and the wells were cleaned with filter paper dipped in methanol, the plates were placed in electrophoresis unit with buffer and load 40µL of the processed protein samples into the wells. The sample was electrophoresed till the dye reaches the base of the gel, the gel was removed carefully and placed in staining solution overnight. The excess stain was removed by placing gel in destaining solution, the proteins bands were visualized.

Enzyme kinetics: factors affecting enzyme activity

Effect of pH on Enzyme Activity

The effect of pH on cellulase activity was determined by incubating the reaction mixture with different buffers of 0.1 molarity (pH 3, 4, 5 – Citrate buffer, pH 6, 7, 8 phosphate buffer and pH 9, 10 – glycine NaOH buffer; Stocks of A and B was prepared for each buffer and the necessary amount was added from each stocks and the pH was adjusted to the desired value and the volume was made up to 20 mL) 1mL CMC prepared in all these buffers is pipetted into test tubes and 0.5 mL of enzyme is also added. Blanks respective for each pH is prepared by pipetting 1 mL of the buffers and 0.5 mL of distilled water. All the tubes were incubated at 37°C for 30 minutes. The cellulase activity was determined by DNS assay.

Effect of Temperature on Enzyme Activity

Optimum temperature for enzyme activity was determined by incubating enzyme substrate reaction mixture at different temperatures (4, 25, 30, 37, 45, 55, 65, 75, 85and 100ºC). 0.25 mL of the enzyme and 1mL of 1% CMC (substrate) dissolved in citrate buffer of pH 7 is pipetted into 7 test tubes. Blank respective for each temperature is prepared with 0.5 mL distilled water and 1 mL CMC. All the tubes are incubated for 15 minutes at different temperatures. After incubation 3 mL DNS reagent is added into all the tubes and kept in boiling water for 15 minutes. The tubes are then cooled and 1 mL of Rochelle's salt is added. The cellulase activity is then determined calorimetrically at 540 nm.

Effect of Activators and Inhibitors on Enzyme Activity

Effect of various metal ions like $CaCl_2$, $MgCl_2$, $HgCl_2$ and EDTA on enzyme activity was determined by using 0.5 mL of enzyme extract, 1 mL of 1% Carboxyl-methyl cellulose solution in citrate buffer of pH 7 and 0.5, 1, 1.5 and 2 mL of 25 mM concentration of various metal ions and chelator. Blank is prepared with 0.5 mL distilled water and 1 mL of CMC solution and all the tubes are incubated at 37ºC for 30 minutes. The cellulase activity is determined by DNS assay.

Biochemical tests

Indole test

20 mL of indole broth with the following composition (g/L) - Tryptone 20g; NaCl 5g; was prepared and 5 mL of broth was dispensed in 3 test tubes. Two tubes were inoculated with the selected isolate under aseptic condition and one tube served as control. All the tubes were incubated at 37°C for 24 h. After incubation, 5 to 6 drops of Kovac's reagent was added and observed for the color change.

Citrate utilization test

50 mL of Simmon's citrate agar with the following composition (g/L) – $MgSO_4$ 0.2g; ammonia 1g; K_2HPO_4 1g; sodium citrate 2g; NaCl 5g; Bromothymol Blue 0.08g; Agar 15g; was prepared and 15 mL of media was poured in 3 test tubes and the loop of colony was streaked on Simmon's citrate agar medium in two tubes under aseptic condition. One tube served as control and all the tubes were incubated at 37°C for 24 h. The experiment was performed in duplicates.

Casein hydrolysis test

50 mL of Casein agar medium with the following composition (g/L) – Skim milk powder 100g; Peptone 5g; Agar 15g; was prepared and 25 mL of media was poured in two petri plates. The loop of colony was streaked on Casein agar medium under aseptic condition, incubated at 37°C for 24 h. The experiment was performed in duplicates.

Gelatin test

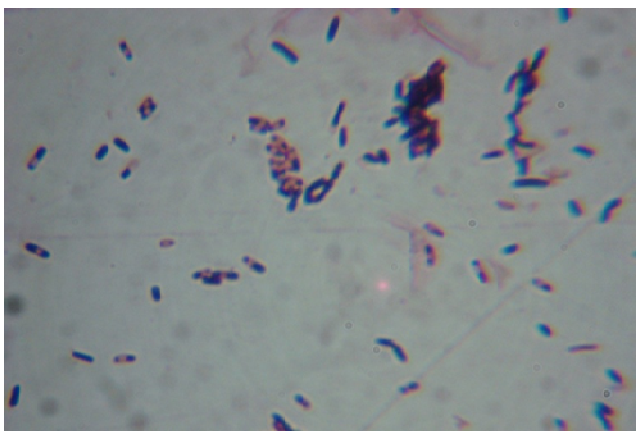
50 mL of Gelatin media with the following composition (g/L) – Beef extract 3g; Peptone 5g; Gelatin 120g; was prepared and 5 mL of broth was dispensed in 3 test tubes. Two tubes were inoculated with the selected isolate under aseptic condition and one tube served as control. All the tubes were incubated at 37°C for 24 h. After incubation, the tubes were kept in deep freezer for 20 minutes and then the results were observed. The experiment was performed in duplicates.

Starch hydrolysis test

50 mL of Starch agar medium with the following composition (g/L) – Peptone 5g; Beef extract 3g; Soluble Starch 12g; Agar 15g; was prepared and 25 mL of media was poured in two Petri plates. The loop of colony was streaked on Starch agar medium under aseptic condition and incubated at 37°C for 24 h. After incubation, iodine solution was added and observed for color change. The experiment was performed in duplicates.

RESULTS

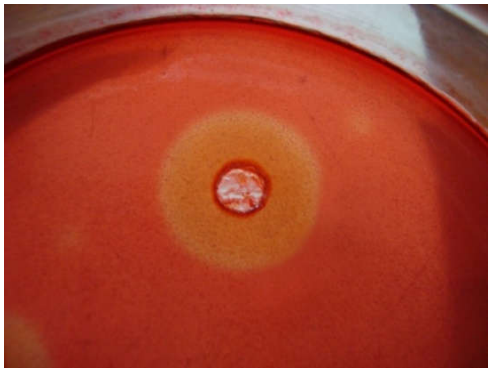
Gram staining



Observation of gram stained bacteria

Colony 1	Gram positive rods, in chains with endospores
Colony 2	Gram positive rods, in chains with endospores
Colony 3	Gram positive rods, in groups
Colony 4	Gram positive rods, in chains with endospores
Colony 5	Gram positive rods, in chains with endospores
Colony 6	Gram positive rods, in chains with endospores
Colony 7	Gram positive rods, in groups with endospore
Colony 8	Gram positive rods, individual
Colony 9	Gram positive rods, in cluster
Colony 10	Gram positive rods,individual with endospore
Colony 11	Gram positive rods, in chains with endospore
Colony 12	Gram positive rods, in groups
Colony 13	Gram positive rods, endospore present
Colony 14	Gram positive rods, in chains with endospores
Colony 15	Gram positive rods, individual
Colony 16	Gram positive rods,individual

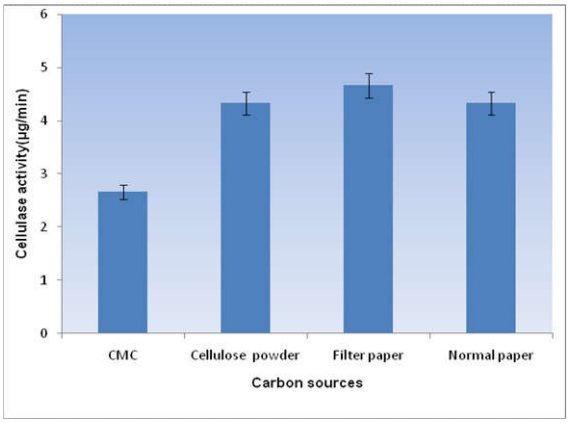
Screening of isolates for cellulose production



Congo red test for cellulase conformation

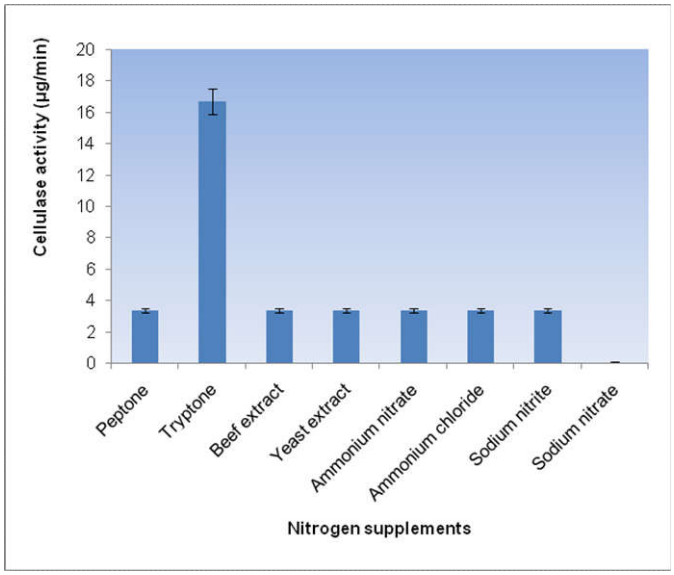
Colony 1	Positive,1.2cm
Colony 2	Negative
Colony 3	Positive,1.6cm
Colony 4	Positive,1.6cm
Colony 5	Positive,1.2cm
Colony 6	Negative
Colony 7	Negative
Colony 8	Positive,2cm
Colony 9	Positive, 1.2cm
Colony 10	Positive, 1cm
Colony 11	Negative
Colony 12	Positive,2.2cm
Colony 13	Positive, 2.4cm
Colony 14	Positive.1.8cm
Colony 15	Positive,1.8cm
Colony 16	Negative

Carbon sources



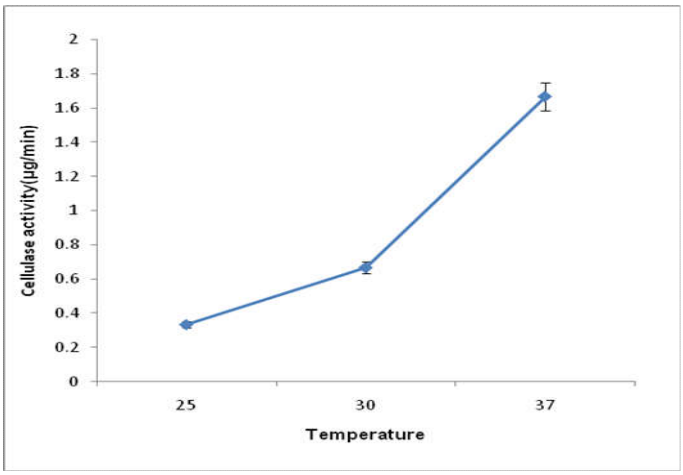
Carbon Sources	Cellulase activity			
	0D	µg/mL	µg/min	per mL
CMC	0.08	40	1.333333	2.666667
Cellulose powder	0.13	65	2.166667	4.333333
Filter paper	0.14	70	2.333333	4.666667
Normal paper	0.13	65	2.166667	4.333333

Nitrogen sources



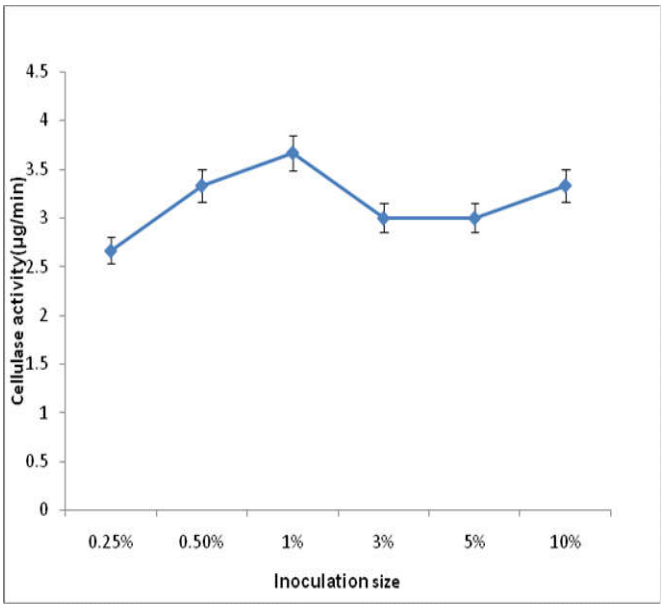
Nitrogen source	OD at 540nm			
	0D	µg/mL	µg/min	per mL
Peptone	0.1	50	1.666667	3.333333
Tryptone	0.5	250	8.333333	16.66667
Beef extract	0.1	50	1.666667	3.333333
Yeast extract	0.1	50	1.666667	3.333333
Ammonium nitrate	0.1	50	1.666667	3.333333
Ammonium chloride	0.1	50	1.666667	3.333333
Sodium nitrite	0.1	50	1.666667	3.333333
Sodium nitrate	0.001	0.5	0.016667	0.033333

Temperature



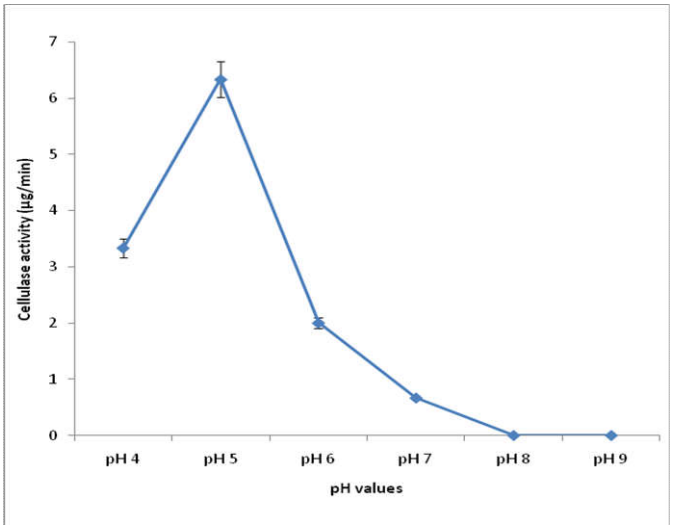
Temperature	OD at 540 nm			
	OD	µg/mL	µg/min	per mL
25	0.01	5	0.166667	0.333333
30	0.02	10	0.333333	0.666667
37	0.05	25	0.833333	1.666667

Inoculation size



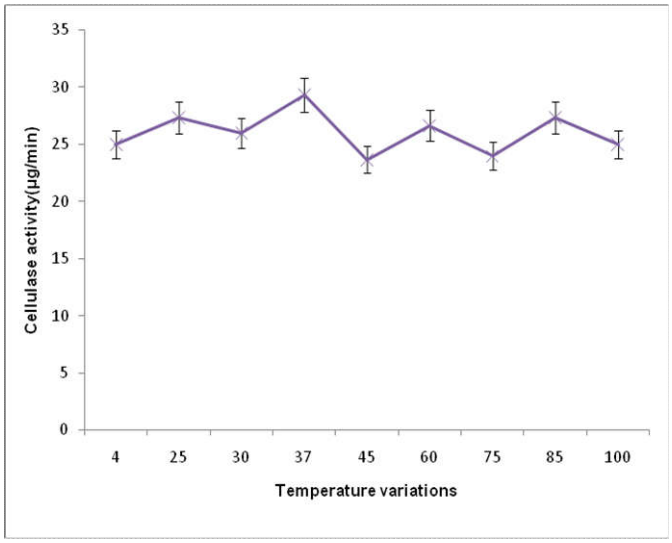
Inoculation Size	OD at 540 nm			
	OD	µg/mL	µg/min	per mL
0.25%	0.08	40	1.333333	2.666667
0.50%	0.1	50	1.666667	3.333333
1%	0.11	55	1.833333	3.666667
3%	0.09	45	1.5	3
5%	0.09	45	1.5	3
10%	0.1	50	1.666667	3.333334

pH



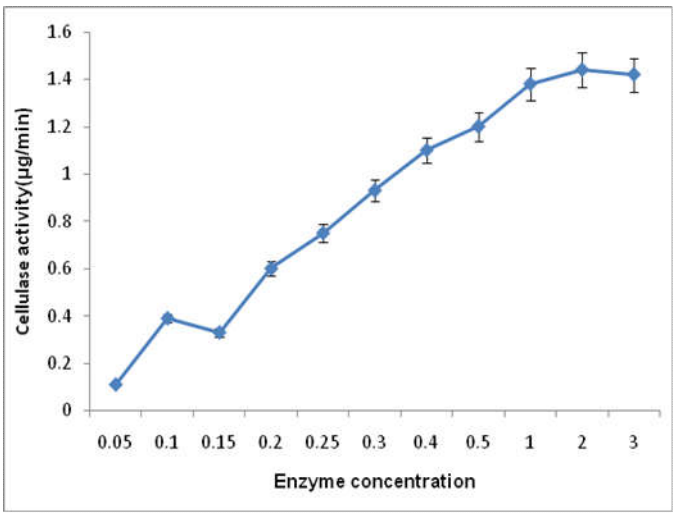
pH	OD at 540 nm			
	OD	µg/mL	µg/min	per mL
pH 4	0.1	50	1.666667	3.333333
pH 5	0.19	95	3.166667	6.333333
pH 6	0.06	30	1	2
pH 7	0.02	10	0.333333	0.666667
pH 8	0	0	0	0
pH 9	0	0	0	0

Temperature variations



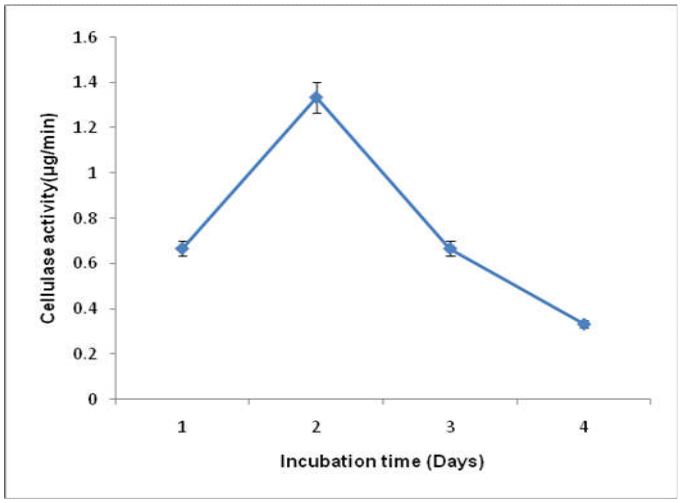
Temperature	OD at 540 nm			
	0D	µg/mL	µg/min	per mL
4	0.75	375	12.5	25
25	0.82	410	13.66667	27.33333
30	0.78	390	13	26
37	0.88	440	14.66667	29.33333
45	0.71	355	11.83333	23.66667
60	0.8	400	13.33333	26.66667
75	0.72	360	12	24
85	0.82	410	13.66667	27.33333
100	0.75	375	12.5	25

Enzyme concentration



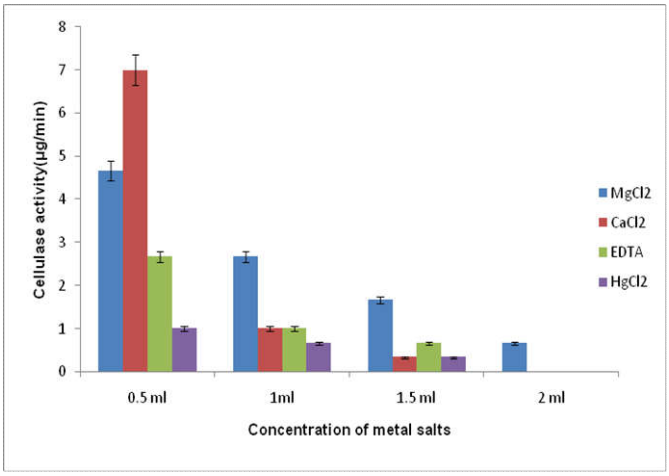
Enzyme concentration	OD at 540nm			
	0D	µg/mL	µg/min	per mL
0.05	0.11	55	1.833333	3.666667
0.1	0.39	195	6.5	13
0.15	0.33	165	5.5	11
0.2	0.6	300	10	20
0.25	0.75	375	12.5	25
0.3	0.93	465	15.5	31
0.4	1.1	550	18.33333	36.66667
0.5	1.2	600	20	40
1	1.38	690	23	46
2	1.44	720	24	48
3	1.42	710	23.66667	47.33333

Incubation time (Days)



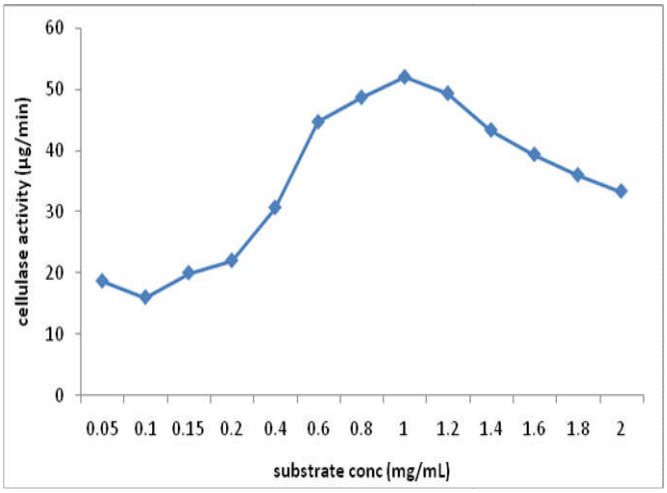
Incubation time (Days)	OD at 540 nm			
	OD	µg/mL	µg/min	per mL
1	0.02	10	0.333333	0.666667
2	0.04	20	0.666667	1.333333
3	0.02	10	0.333333	0.666667
4	0.01	5	0.166667	0.333333
5	0	0	0	0

Concentration of metal salts



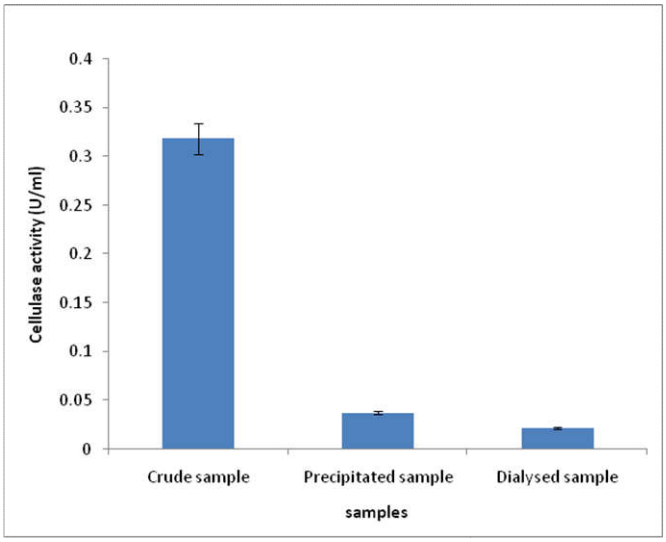
Effect of salts		OD at 540 nm			
		OD	µg/mL	µg/min	per mL
Mgcl2	**0.5 mL**	0.14	70	2.333333	4.666667
	1mL	0.08	40	1.333333	2.666667
	1.5 mL	0.05	25	0.833333	1.666667
	2 mL	0.02	10	0.333333	0.666667
Cacl2	**0.5mL**	0.21	105	3.5	7
	1mL	0.03	15	0.5	1
	1.5mL	0.01	5	0.166667	0.333333
	2mL	0	0	0	0
EDTA	**0.5mL**	0.08	40	1.333333	2.666667
	1mL	0.03	15	0.5	1
	1.5mL	0.02	10	0.333333	0.666667
	2mL	0	0	0	0
Hgcl2	**0.5mL**	0.03	15	0.5	1
	1mL	0.02	10	0.333333	0.666667
	1.5mL	0.01	5	0.166667	0.333333
	2mL	0	0	0	0

Substrate concentration



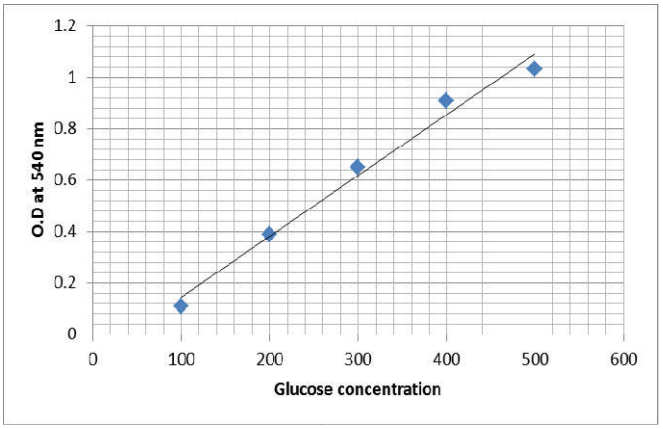
Substrate concentration mg/mL	OD at 540 nm			
	0D	µg/mL	µg/min	per Ml
0.05	0.28	140	4.666667	18.66667
0.1	0.24	120	4	16
0.15	0.3	150	5	20
0.2	0.33	165	5.5	22
0.4	0.46	230	7.666667	30.66667
0.6	0.67	335	11.16667	44.66667
0.8	0.73	365	12.16667	48.66667
1	0.78	390	13	52
1.2	0.74	370	12.33333	49.33333
1.4	0.65	325	10.83333	43.33333
1.6	0.59	295	9.833333	39.33333
1.8	0.54	270	9	36
2	0.5	250	8.333333	33.33333

Specific activity of enzyme



Specific activity	OD at 540 nm	
	µmol/min	µmol/min/mg
Crude sample	0.249778	0.318594
Precipitated sample	0.009251	0.037545
Dialysed sample	0.0037	0.022026

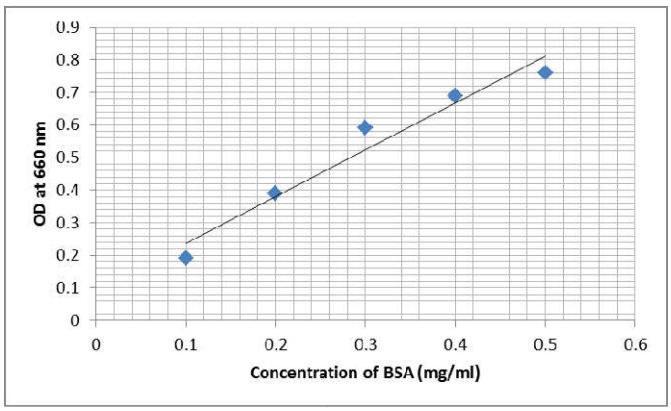
Standardization of glucose



Standard Glucose Chart

Sl. No	Glucose Standard in mL	O.D at 540nm
1	0.2	0.11
2	0.4	0.39
3	0.6	0.65
4	0.8	0.91
5	1.0	1.03

Standardization of BSA



Standard BSA chart

Sl. No.	BSA(mg/mL)	OD
1	0.1	0.19
2	0.2	0.39
3	0.3	0.59
4	0.4	0.69
5	0.5	0.76

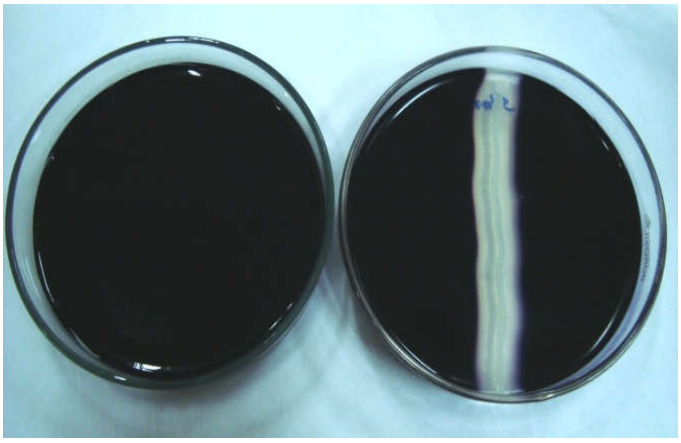
Biochemical Test Results

Casein hydrolysis test (Positive)

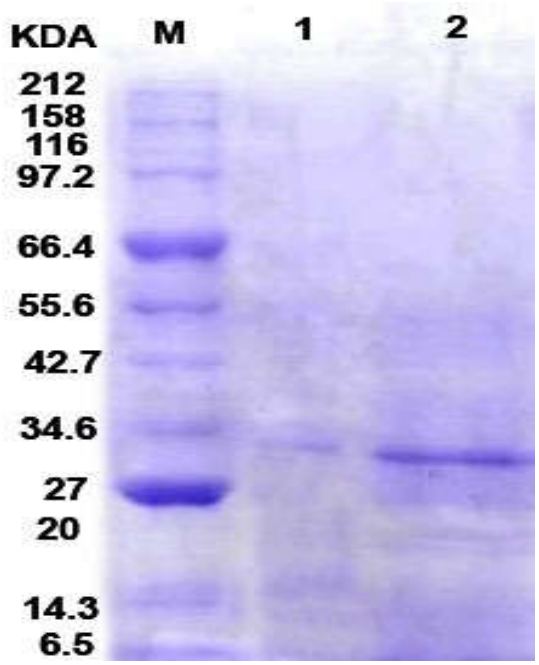
Citrate utilization test (Positive)

Gelatin test (Positive)

Indole test (Negative)



Starch hydrolysis test (Positive)



SDS – PAGE gel

The apparent molecular weight of partially purified cellulase from bacillus sps. was found to be 32 KDa by SDS – PAGE.

DISCUSSION

This study could establish that paper industrial waste, timber saw mill waste which does not find any significant commercial use especially in developing countries like India and is disposed of in municipal bins for rotting could serve as an ideal substrate for production of cellulases. Hence, the technology using these cheap and readily available substrates for production of cellulase in optimum quantities in promise for the future. The results are significant for the study on cellulase production and provide a potential approach for the industry. The cultural conditions were optimized for higher yield of cellulase enzyme. For the initial optimization of the medium, the traditional method of “one variable at a time” approach was used by changing one component at a time while keeping the others at their original level. Thus the present investigation was selected to conduct an extensive study on

cellulase from *Bacillus* species. Present study was aimed at isolation of promising cellulase producing *Bacillus* sp. and its identification, optimization of cultural conditions for production of cellulase enzyme. After the bacterial isolation each of the bacterial strains were analyzed for its potential to produce cellulolytic enzyme employing a simple qualitative Congo red dye based method. The samples containing bacteria were inoculated on a medium containing CMC (0.5% w/v). The plates were incubated overnight at room temperature and the medium was flooded with Congo red dye solution., cellulase produced by bacteria hydrolyzed the substrate(CMC) incorporated in the medium and the dye Congo red did not stain the region where the CMC is hydrolyzed. Congo red stained the unhydrolyzed CMC incorporated in the medium. Hydrolysis of CMC clearly suggests that bacteria produced cellulase outside the cells. The hydrolyzed regions appear somewhat transparent. A total number of 16 bacterial strains were isolated from rhizosphere soil on nutrient agar plate. Of the 16 isolates only one strain potentially hydrolyzed CMC in the medium. Therefore, this particular bacterial strain was chosen and identified as *Bacillus sps.* and maintained in agar slants. The enzyme activity when supplemented with various carbon sources was found that, the filter paper produced the more activity. Glucose was found to be the best carbon source for the cellulose production (Shanmugapriya *et al*., 2012).The enzyme activity when supplemented with various nitrogen sources was found that, tryptone gave more activity compared with others sources. Yeast extract showed maximum activity when the bacterium was grown for 72 h (Sakthivel *et al.,* 2010) The production of cellulase enzyme was found more in the pH 5. Results of enzyme assay by Iqbal *et al*., 2011 showed that the cellular enzyme was completely active in a large pH range (5 -8) and presented an optimum activity of 195U/mL at a pH value of 8 which was a little higher than those from *Mucorcircinelloides* 4 - 7 and Bacillus circulars, 4.5 - 7. Whereas any further increase in pH from optimum value (pH 8) cellulase showed decreasing trends in activity. This little variation in pH optima may be due to genetic viability among different species. The cellulase production was found to be more when incubated for two days (48 hrs).The production was found to be more when the inoculation size was 1%.The production was found to be more in citrate buffer than phosphate buffer and glycine NaOH buffer. The substrate concentration at 1% showed maximum production of the cellulase enzyme. Maximum production of the cellulase enzyme by using different metal ions. The metal ions used were $HgCl_2$, EDTA, $CaCl_2$ and $MgCl_2$. $CaCl_2$ showed more enzyme activity when compared with the others taken for optimization. For the production of the cellulase enzyme the optimum temperature was found to be at 37˚C. The production of the enzyme was maximum at 2 mL of enzyme concentrations. The od 0.5 corresponds to 0.28 mg/mL from the Lowrys method estimated for the enzyme. The specific activity of the enzyme was found to be 0.3 mg/mL. The apparent molecular weight of partially purified cellulase was found to be 32 KDa by SDS – PAGE.

Conclusion

The present work was carried out to optimize the nutritional and environmental parameters for improving cellulase production by the cellulolytic bacteria. The cellulase producing *Bacillus species* was isolated from rhizosphere soil and characterized by various staining procedures, biochemical analysis and partial purification. From my present study, the result showed that cellulase producing bacteria can grow at optimized condition. Thereby, partial purification of cellulase enzyme was done and determined molecular weight of the enzyme. The *Bacillus* species showed a potential to convert cellulose into reducing sugars which could be readily used in many applications such as animal foods and a feed stock for production of valuable organic compounds. The use of microorganisms for the production of enzymes offers a promising approach for its large scale production and as a possible food supplement or in pharmaceutical industry. Thus the present investigation was selected to conduct an extensive study on cellulase from *Bacillus* species. Present study was aimed at isolation of promising cellulase producing *Bacillus* sp. and its identification, optimization of cultural conditions for production of cellulase enzyme. Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials.

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