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Processing, Optimization, partial Purification, and Application of Bacterial Cellulase from Agro-waste

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ABSTRACT
Cellulase is a hydrolytic enzyme which breaks down cellulose into smaller oligosaccharides and glucose. A total of 100 samples were collected from different sites of Allahabad, of which 40 gave positive result for cellulase production. The positive isolates were screened and selected on the basis of maximum hydrolytic zone formation on CMC agar media. The organism that gave maximum cellulase production was identified as *Bacillus cereus, Bacillus Pantothenticum, Bacillus Insolitus, Bacillus alvei, Bacillus Firmus, Bacillus alvei, Bacillus Alcolphilus, Bacillus Alvei* and *Bacillus subtilus*. Cellulase was produced by *Bacillus cereus* with sugarcane bagasse as solid substrate. The different parameters for cellulase production were evaluated and it was observed that the maximum production was found at 72hrs incubation period, 30°C, 7.5pH, 1%lactose additional carbon source, 1%peptone additional nitrogen source. Crude enzyme was partially purified by ammonium sulphate precipitation, dialysis and DEAE-cellulose ion-exchange chromatography. The total activity of crude enzyme and total activity of chromatography enzyme showed a 4537.2 and 323.52 respectively. A total protein content of crude and total protein content of ion-exchange chromatography showed a 1633.17mg/ml and 77.26mg/ml. DEAE-cellulose chromatography resulted in purification fold is 1.50. The effect of pH and temperature on cellulase activity was characterized, enzymatic activities were found at7.0 pH (5.86U/ml) and at 50°C (40.12U/ml). Compatibility of cellulase enzyme with different detergent brands in which maximum compatibility were examined for Tide detergent (enzyme activity 81.78U/ml).]

Keywords: *Bacillus cereus*, cellulase, DEAE-cellulose chromatography ,solid State Fermentation, submerged Fermentation, Carboxy methyl Cellulose, colony forming unit, total sum of square, Error sum of square, Error mean sum of square, Analysis of variance test, Rotation per minutes, Degree of freedom

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I. INTRODUCTION:

Cellulases refer to a class of enzymes produced chiefly by fungi, bacteria and protozoan that catalyze the hydrolysis of cellulose. Unlike chemical methods, enzymatic hydrolysis of lignocelluloses offers an attractive method and relatively pure products can be obtained from the hydrolytic process. Such products can serve as raw material for the production of bio-ethanol, glucose and a few other compounds **Solomon et al., (1999); Dashtban *et al.*, (2009).** Cellulose is the major component of plant biomass and the major biopolymer found in abundance on earth, and much of the cellulose exists as wastes. Such wastes include straw, corn cobs, wood wastes, peat, bagasse and waste paper **Ojumu et al., (2003)** .In principle, all lignocellulosic can be converted into simple sugars which can serve as useful raw materials in the production of fuel, animal feedstock and feedstock for chemical synthesis **Bhat. (2000)**. Against this backdrop, there has been several studies on the use of different agricultural wastes which include bagasse **Solomon*et al.*,(1999); Ojumu *et al.*,(2003),** corn cob **Ojumu *et al.*,(2003); Betini *et al.*,(2009),** sawdust**Lo *et al.*,(2005); Chinedu *et a.*,(2008),** wheat bran **(Betini *etal* 2009)** and wheat straw **Yang *et al.*,(2006)** as lignocellulosic substrate for cellulase hydrolysis. Cellulose present in renewable lignocellulosic material is considered to be the most abundant organic substrate on earth for the production of glucose, for fuel and as chemical feed stock. Research on cellulase has progressed very rapidly in the past few decades, emphasis being on enzymatic hydrolysis of cellulose to glucose. The

enzymatic hydrolysis of cellulose requires the use of cellulase [1,4-(1,3:1,4)-b-D-glucanase glucanohydrolase, EC 3.2.1.4], a multiple enzyme system consisting of endo 1,4,-b-D-glucanases [1,4-b-D-glucanases (CMCase, EC 3.2.1.4)], and exo 1,4,-b-D-glucanases [1,4-b-D glucanase cellobiohydrolase, FPA, EC 3.2.1.91] along with cellobiase (b-D-glycoside glucanohydrolase, EC 3.2.1.21).Major impediments to exploiting the commercial potential of cellulases are the yield, stability, specicity, and the cost of production **Bhat and Bhat, (1997).** In the past few decades focus has been on submerged fermentation (SmF) and very little attention has been given to solid-state fermentation (SSF). The direct applicability of the product, the high product concentration, and the reduced costs of dewatering make SSF a promising

Technology for cellulase production **Vandevoorde and Verstraete, (1987).** Production of cellulase in SSF using various substrates, microorganisms, and nutrient solutions has been reported (Shamala and Srikantaiah, 1986; Macris et al., 1989; Muniswaran and

Charyulu, (1994). A cellulolytic enzyme system is a multi-enzyme for desirable responses and reducing the number of system comprising of endoglucanase (endo-1,4- -D- required experiments [10]. In biological processes, glucanase, EC 3.2.1.4), exo-glucanase (1, 4- -D-glucanase- especially in the production of ethanol from biomass, cellobiohydrolase, EC 3.2.1.91) and -glucosidase RSM has been adopted to optimize the growth of (D-glycoside glucanohydrolase, cellobiase, EC microorganisms and the production of ethanol that acts synergistically to degrade cellulosic 12]. In this work, RSM was adopted to determine the Substrate **Holker *et al.*,(2004**). Cellulolytic enzymes are key to biomass optimal conditions for the production of cellulase from processing for the production of fuel ethanol and *Bacillus pumilus* EWBCM1 and the interactions among byproducts. High cost of these enzymes, however, is a factor that influences the response of the cellulose significant barrier to the commercialization of ethanol and production. Chemicals. Due to the heterogeneity and complexity of lignocellulosic biomass. **Esterbauer *et al.*,(1991)**. It is well known that plants are the most common source of renewable carbon and energy on the earth. They annually produce about 4 □ □ 109 tons of cellulose which is a highly stable polymer consisting of β-1, 4-linked glycosyl residues, along with other polysaccharides **Coughlan *et al.*,(1990)**. The potential of these biological resources are possible substitutes for diminishing fossil energy resources and becoming increasingly important.

The biological degradation of cellulose has been studied for many years, and a number of cellulolytic enzymes, especially cellulases produced by fungi and bacteria, have been isolated and characterized **Tomme *et al.*,(1995).** These enzymes, which cleave the

β-1, 4 bond of cellulose, belongs to the large family of glycosyl hydrolases. On the basis of sequence comparisons and hydrophobic cluster analysis, the catalytic domains of all glycosyl hydrolases have been classified into 63 families of homologous folds **Henrissat *et al.*,(1997); Bairoch and Henrissat, (1993); Henrissat and Bairoch (1996).**

The catalytic domains of cellulase and related xylanase have been identified in 14 of these families. According to their mechanism of cellulose degradation, cellulases are subdivided into either non-processive cellulases (Endocellulase) or processive cellulases (including different exocellulases and some new processive Endocellulase)

Barr *et al.*, (1996); Reverbel *et al.*,(1997.). Endocellulases can randomly cleave the cellulose at exposed positions and produce new reducing ends, however, the processive cellulases remain attached to the chain and release mainly cellobiose or cellotetraose units from one end of the chain **Sakon *et al.*,(1997).** These mechanisms are similar to that degradation of amylase **Robyt and French (1967).** During the degradation of cellulose, non-processive cellulases and processive cellulases have been found to work synergistically **Creuzet *et al.*,(1983); Henrissat *et al.*,(1985).** It has been generally accepted that effective biological hydrolysis of cellulose into glucose requires synergistic actions of three enzymes including endo-β-1, 4-glucanase (EC 3.2.1.4, EG, randomly cleaving internal linkages), cellobiohydrolase (EC

3.2.1.91, CBH, specifically ***et al.,*** hydrolyzing cellobiose units from non-reducing ends), and β-*D*-glucosidase (EC 3.2.1.21, hydrolyzing glycosyl units from cellooligosaccharides) **Perez (2002).** Currently, cellulase is commonly used in many industrial applications, especially in animal feed, textile, waste water, brewing and wine-making **Beguin and Aubert (1994).** With the shortage of petroleum fuels, increase of greenhouse gases and air pollution due to the incomplete combustion of fossil fuel, there has been increasing worldwide interest in the production of bioethanol from lignocellulosic biomass **Zaldiver *et al.*, (2001).** To utilize these materials and to avoid waste pollution, one of the most important approaches is to find applicable cellulase and hemicelluloses to hydrolyze the lignocellulosic biomass. The cost of related enzymes has been considered to be critical to the success of bioethanol industry. Accordingly, a highly stable, efficient and also economic cellulase or hemicelluloses for the bio-fuel industry has been

attracted many scientists concerns. Among bacteria, *Bacillus* species can produce numbers of extracellular polysaccharide hydrolyzing enzyme **Bhatt and Bhatt (1997).** Cellulose is the major component of plant biomass **camasola and Dillon (2007).** Plants produce 4×109 tons of cellulose annually **Couyhlan M. (1990).** It is a polymer of β-1, 4 linked glucose units. Its crystalline structure and insoluble nature represents a big challenge for enzymatic hydrolysis.

Microorganisms are important in conversion of lignocellulosic wastes into valuable products like biofuels produced by fermentation **Lynd *et al.*,(2002).** Successful bioconversion of cellulosic materials mainly depends on the nature of cellulose, sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes**Alam *et al.*,(2004).** Cellulose quality, temperature, aeration, carbon sources, incubation period, medium additives, pH of the medium and presence of inducers are important parameters for the optimized production of cellulase enzymes **Immanuel *etal.*, (2006).** For many years, cellulose degrading bacteria have been isolated and characterized for obtaining more effective cellulases from variety of sources such as soil, decayed plant materials, hot springs, organic matters, feces of ruminants and composts **(Doi 2008).** Researchers keep on working to isolate microorganisms with higher cellulase activity **Ray *et al.*,(2007).** A cellulolytic enzyme system is group ofenzymes that work synergistically to hydrolyze lignocellulosic biomass. It is composed ofendoglucanase (endo-1, 4-β-D--glucanase, EC 3.2.1.4), exoglucanase (1, 4-β -D-glucanase cellobiohydrolase, EC 3.2.1.91), and β-glucosidase (β-D-glycoside glucanohydrolase,cellobiase, EC 3.2.1.21) **Holker *et al.*, (2004)**; **Esterbauer *et al.*,(1991).** Xylanase (EC 3.2.1.8),though not part of the group, complements the cellulolytic enzyme system as it is needed toelicit complete and efficient hydrolysis of the lignocellulosic biomass, which has anappreciable amount of hemicelluloses or xylan **Brijwani *et al.*,(2010); Brijwani, (2011).** It hasbeen widely accepted that Solid State Fermentation (SSF) is an attractive means to producecellulase economically because of its lower capital investment and lower operating cost **Cenand Xia (1999).** Further, the ability of SSF to minimize catabolism repression has been alreadydescribed for several enzymes **Aguilar and Huitron (1986); Archana and Satayanaryana (1997);Siqueira *et al.*,(1997); Solis-Pereyra (1996).** SSF is defined as a fermentation process in whichmicroorganisms grow on solid materials without the presence of free liquid and themoisture necessary for microbial growth exists in adsorbed state or complexes with solidmatrix **(Krishna, 2005).** Both bacteria and fungi are known to produce cellulases using complex cellulosic substrates, however, fungal enzymes are generally completecomprising of all the cellulosic activities **Stockton *et al.*, (1991)**. The operating conditions like temperature, pH and moisture content are very important for microbial growth and efficient cellulase production in SSF. Optimization of parameters in multifactor experimental designs fall short in locating true optimum especially when there are interactions among independent variables, besides being time consuming **Giovanni (1983);Theodore and Panda (1995).** Aguilar G, Huitron C. Application of fed-batch treatments in the production of extracellular pectinase by *Aspergillus sp*. Enzyme Microbe Technol 1986; Stockton BC, Mitchell DJ, Grohmann K, et al. Optimum β-D glucosidase supplementation of cellulase for efficient conversion of cellulose to glucose. Biotechnology Lett 1991.Cellulose, an abundant and renewable energy resource,can be converted into useful products such as solublesugars, alcohols and other industrially important chemicalsby enzymatic degradation **Ryu and Mandels, (1980);Mandels, (1985).** Cellulolytic microorganisms are foundamong extremely variegated taxonomic groups. Mostbelong to eubacteria and fungi, such as aerobic andanaerobic bacteria **Gilkes *et al.*, (1991)**, white rot fungi **Uzcategui *et al.*, (1991),** soft rot fungi **Wood *etal.*, (1988)** and anaerobic fungi **Barichievic and Calza,(1990),**however, anaerobic, cellulose-degrading protozoa havealso been identified in the rumen **Coleman, (1978).**Microorganisms capable of hydrolyzing this biopolymersecrete cellulase, include three types of enzymes,namely endo-1,4-β-D-glucanase [(1, 4)-β-D-glucanase glucanohydrolase,EC3.2.1.4], exo-1,4-β-D-glucanase [(1, 4)-β-D-glucanase cellobiohydrolase, EC3.2.1.91] and β-glucosidase [β-D-glycoside glucohydrolase, EC3.2.1.21](Wood, 1985).Cellulases have attracted much attention because oftheir diverse practical applications and the need tounderstand the mechanisms of their hydrolysis of plantcarbohydrate polymers **Bhat and Bhat, (1997).** The majorindustrial applications of cellulases are in the textileindustry for "bio-polishing" of fabrics and producingstonewashed look of denims, as well as in householdlaundry detergents for improving fabric softness andbrightness **Cavaco-Paulo, (1998).** Besides, they are usedin animal feeds for improving the nutritional quality and digestibility, in the processing of fruit juices and in baking. De-inking of paper is yet another emerging application **Tolan and Foody, (1999).D**ue to the above novel applications and advantages, the present study entitled **"Production, Optimization, partial Purification and application of bacterial cellulase from agro-**

waste" and was be undertaken with the following objectives:

1. To screen and identify the Cellulase producing bacteria from soil.
2. To optimize the production parameters for cellulase production.
3.Topartiallypurify andcharacterize cellulase enzyme.
4. To Study the compatibility of cellulase with different detergent brands.

II. MATERIAL &METHODS

Place of Work: The study on "Processing , Optimization, Partial Purification and Application of Bacterial Cellulase from Agro-waste" was carried out in the School of Bio-Engineering and Biotechnology, Sam Higginbottom Institute of Agriculture Technology and Sciences, (Deemed to be University) Allahabad.

Collection of sample

Soil Sample was collected from different places of Allahabad in sterile bottle. A total of 100 soil samples were collected from five different sites of Allahabad region of which 20 samples each from Garden soil, wood furnishing regions, Sugar cane farm soil, Petrol pump, Chimney regions.

Isolation of Cellulosic Bacteria from Soil

For the Isolation of bacterial load; 1gm soil sample was dissolved in 9 ml of sterile Ringer solution (App) and this suspension was serially diluted up to10^{-5} and 10^{-6}. 1ml of diluted suspension was transferred to Petri Plate and then carboxyl methyl cellulase (CMC) Agar medium (App) was poured over it and incubated for 48 hrs at 37±1ºC. **(Nakamura et al., 1984).**

Screening and Selection of Cellulolytic Strain

After incubation at 37±1 ºC for 48 hrs, the agar media were flooded with an aqueous solution of Congo red (1%W/v) for 15 min. the Congo red solution was then poured off, and plates were further treated by flooding with 1M NaCl for 15min. The formation of clear zone of hydrolysis indicated cellulose degradation. The strain thus showing maximum cellulase activity within short period of time was selected for further studies.

Identification of strain

For the identification of strain of interest cultural characterization, morphological characteristics and biochemical tests were conducted and identified on the basis of characters as given in bergey's Manual of systematic Bacteriology.

Cultural Characteristics

The isolate was identified based on different cultural characteristics like color, texture, margin, elevation, density and size on culture plates.

Morphological Charactiscs

Gram staining of isolated bacteria was done and observed under 100X objective of microscope. This was done for identifying and classifying bacteria as gram positive or gram negative.

BIOCHEMICAL TESTS

The biochemical identification of the isolates was done as per the procedure given by **Aneja, (2013).** The procedures for all biochemical tests are described below:

SUGAR FERMENTATION

Fermentation degradation of various carbohydrates such as D-glucose, C-Arabinose, D-Xylose, D-Mannose etc, by microbes under aerobic condition was carried out in sugar tubes having Durham's tubes for the detection of gas production as an end product of metabolism. The sugar tubes were contained ingredients of basal broth (4.0 ml)(App. I 1.3); a specific carbon (0.5ml) and a pH indicator (Phenol red) which was red at neutral pH but turns yellow at pH below 6.8 due to the production of an organic acid.

CATALASE TEST

Catalase test was performed to determine whether the organism is able to produce oxygen gas, during aerobic respiration, in the presence of oxygen, microorganism produces hydrogen peroxide which is lethal to cell. Catalase test was performed by adding hydrogen peroxide solution to culture on a slide. The enzyme catalase present in the (App.1.15); organism breaks down hydrogen peroxide to water and oxygen. The presence or absence of free oxygen bubbles was found on the slides, which gives positive catalase test.

METHYL RED TEST

The methyl red test involves adding the pH indicator methyl red to MR-VP broth (App). If the organism uses the mixed acid fermentation pathway and produces stable acidic end products, the acid overcome the buffers in the medium and produces an acidic environment in the medium. When methyl red indicator (App. II 2.2) was added if acidic end products are present, the methyl red was remaining red.

VOGES-PROSKAUER TEST

This test was performed to determine the capability of microorganism to produce non-acidic end products such as ethanol and acetoin (acetyl methyl Carbinol) from the organic acid. The isolated microorganism was inoculated in MR-VP broth. All the inoculated and uninoculated tubes were incubated at 37±1ºC for 24-48 hrs. After incubation 15 drops of freshly prepared VP-reagent I (Naphthol solution) (App. II 2.3) and 2-3 drops of VP-reagent II (40% KOH) was added in all the inoculated and

uninoculated tubes. Development of Crimson to pink (red) color may be most intense at the surface, which indicates positive test while no change in color indicates negative test.

CITRATE UTILIZATION TEST

Citrate utilization test was performed to determine the ability of microorganism to utilize citrate as carbon source. The utilization of citrate depends on the presence of an enzyme citrate that breakdown citrate to oxaloacetic acid and acetic acid. The isolated microorganism was inoculated in simmon's citrate agar (App. II 1.9) slant and incubated at 37±1 ºC for 24-48 hours. After incubation, tubes were examined change in coloration of slant from green to blue indicates positive test for citrate utilization. If colour does not change, it indicates negative test for citrate utilization.

INDOLE-FORMATION TEST

This test was performed to find out whether the organism was able to oxidize tryptophan into Indole, Pyruvic acid and ammonia. The isolated organism was incubated into tryptone broth (App.1.7). The Inoculated and uninoculated tubes were incubated at 37±1 ºC for 24-48 hours. After incubation, kovac's reagent (Dimethyl amino benzaldehyde)
(App. II 2.1) was added to inoculated and control tubes. Resultant of cherry red color at the top layer in the front of ring indicate positive test while its absence indicates Negative reaction for Indole production.

GELATIN HYDROLYSIS TEST

Gelatin hydrolysis test was performed on gelatin agar medium (App. 1.17) which was prepared and autoclaved at 15 lbps for 15-20 min. Then stab inoculation was done in culture tube by sterile needle and incubated at 37±1ºC for 24-48 hours. After that tubes were kept in the refrigerator for 15min and compared with the control tube.

STARCH HYDROLYSIS TEST

The starch agar (App. II 1.12) plate was streaked with the isolated organism in sterile condition. After that the plates were incubated at 37±1ºC for 24-48 hrs Inverted position. After incubation the surface of plates was flooded with iodine solution, with dropper for 45 second. The plates were examined for the color change of the medium. A clear zone surrounding the microbial colonies indicates a typical positive starch hydrolysis while a negative reaction shows dark blue coloration.

OXIDASE TEST

It was done by inoculating culture on Wattmaan's filter paper to which Oxidase reagent (N, N, N, N, -tetramethyl-Para-Phenylenediamine
dihydrochloride) (App. 1.14) was added and change in color from brown to purple was examined for Oxidase production.

LITMUS MILK TEST

Litmus milk broth (App. 1.19) was prepared and autoclaved 15 lbps for 15-20 min. Then inoculation was done in culture tube by sterile needle and incubated at 37±1ºC for 24-48 hours. After that tubes were keeping observed and compared with the control tube.

UREASE TEST

InUrease test many organisms especially those that infect the urinary tract, have a urease enzyme, which is able to split urea in the presence of water to release ammonia and carbon dioxide the ammonia combined with carbon dioxide and water to form ammonium carbonate. This terms the medium alkaline, turning the indicator phenol red from its original orange yellow colour to bright pink. The urea broth was autoclave at 10lbs for 10 minutes. Then inoculation was done in culture tube by sterile needle and incubated at 37±1ºC for 24-48 hours (App.1.16). After that tubes were keeping observed and compared with the control tube.

MOTILITY TEST

Motility test was performed using a sterile needle a well-isolated colony was picked and stabbed into the medium within 1cm of the bottom of the tube. The needle was entered through same line as it entered as it is removed. The tube were incubated at 37ºC for 24 hours or until growth is evident (App.1.10). A positive motility test is indicated by a diffuse cloud of growth away from the line of inoculation and a negative one along the line of inoculation.

TRIPLE SUGAR IRON AGAR TEST

Triple sugar iron agar test was used triple sugar (Lactose, Sucrose and glucose) and also iron. Lactose, sucrose and glucose in the concentration of 10:10:1(i.e. 10 part Lactose (1%), 10 part Sucrose (1%) and 1 part Glucose (0.1%)). Ferrous sulfate use as a indicator of H2S formation (App. 1.18) and also add phenol red indicator of acidification. Triple sugar iron agar test a sterilized straight inoculation loop to streak on the surface of the agar slant through isolated colony and then incubated at 37±1ºC for 24hrs.

SUBSTRATE AND ITS PRETREATMENT

Different Agrowaste substrates i.e. were collected and dried in sun heat. After drying the Agrowaste were then grinded in powder form and stored in polyethylene bags at room temperature (30±1ºC) until use.

PREPARATION OF INOCULUM

Firstly prepared the solution of 100ml Nutrient Broth and sterilized at 121ºC for 15-20min and let it for cool down and was inoculated under aseptic conditions with our isolate, from a nutrient slant. The broth culture was incubated for 24 hrs on a rotary shaker (200rpm) at 30 ºC. Then Inoculation adds 0.5ml culture in the agro waste and mixes it well and incubates it for 72 hrs for 30±1ºC.

PRODUCTION OF CELLULASE PRODUCTION MEDIUM AND CONDITIONS FOR CONTROL

The bacterial strain was allowed to grown in 250ml Erlenmeyer flasks containing each of various substrates (Sugarcane Bagasse, Rice Straw, Chana Husk, Wheat Straw and Ground Nut Shell) and moistened with mineral salt solution (gL-1 $Na_2HPo_4.2H_2O$,1.1; $NaH_2Po_4.2H_2O$, 0.61; KCl, 0.3, $MgSo_4.7H_2O$,0.1; pH 7.0). It had an initial moisture content and was autoclaved at 121ºC for 60 min, cooled to about 30±1ºC and inoculated with an inoculum of 4.5ml. The contents of the flasks were mixed thoroughly to ensure uniform distribution of the inoculum and were incubated at 37±1ºC for 72 hours. The enzyme was then extracted and assayed.**Ajay *et al.*,(2012)**

ENZYME EXTRATION

By using 0.01M phosphate buffer the enzyme was extracted from fermented substrate and adopting simple contact method the 1:10 ratio of phosphate buffer was applied. The extracted enzyme was centrifuge at 8000rpm for 20 min at 4±1ºC and lastly obtained the clear supernatant was used for enzyme assay.**Ajay *et al.*,(2012)**

CRUDE ENZYME ASSAY

At the time of enzymatic reaction by using 3, 5-dinitrosalicyclic acid method reducing sugar was produced and then by estimating this reduced sugar, the enzymatic activity of crude enzyme was determined. Under the described condition per minutes one unit of activated enzyme required to release 1.0µmol of reducing sugar (as glucose)**.** The 1.7 ml of CMC substrate solution was added in a 0.3 ml of crude enzyme (sample solution) and let it boil to 100ºC for 10min. after that removed test tube from water bath and add 3ml DNS solution and again let it boil at 50 ºC for 30 min and settle down to cool. The absorbance at 550nm against water blank was determined. Glucose standard curve was prepared by making different concentration of glucose from 0.1-1.0 gram/ml. then 1ml of glucose solution was taken and 3ml of DNS reagent was added. The mixture of 3ml was boiled for 30 min. till brown colour develops and absorbance was determined at 550nm. Amount of reducing sugar in the unknown sample was calculated from the line regression equation of glucose standard curve and enzyme activity was calculated as follows:-

$$\text{Cellulase activity (g/ml)} = \frac{\text{Amount of reducing sugar (X)} \times \text{dilution factor}}{\text{Molecular weight of glucose} \times \text{time} \times \text{volume of sample}}$$

OPTIMIZATION OF PRODUCTION PARAMETER

The optimized enzyme over a wide range that produced by the influencement of various process parameters during solid state fermentation and the produced enzyme were taken in triplicates for the optimization in standardized parameters.

EFFECT OF INCUBATION PERIODS

Cellulase production was determined at optimum incubation period (37±1ºC) by adding in 10 grams Agrowaste substrate with mineral salt medium with pH 7.0 after transferring 1ml fresh inoculum. The samples were withdrawn at different time intervals (0, 24, 48, 72, 96 and 120 hours) and each time the enzyme activity was assayed by measuring the concentration of reducing sugars formed by enzymatic hydrolysis of carboxymethyl cellulose (CMC).

EFFECT OF INITIAL pH

The mineral salt medium with 0.01N NaOH or 0.01N HCl at different pH (In between 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0). The maximum cellulase production was determined by mixing in 10 grams Agrowaste substrate and the enzymatic activity was determining by measuring the concentration of reduced sugar by incubated media at 30±1ºC with 1ml fresh inoculum formed by enzymatic hydrolysis of carboxyl methyl cellulose.

EFFECT OF TEMPERATURE

1ml fresh inoculum was inoculated in mineral salt medium by mixing it with each 10 grams Agrowaste substrate at different temperature (In between 25…50ºC) for 72 hours, to determining the maximum cellulase production by the hydrolysis process carboxyl methyl cellulose was formed. Which was used to measure the concentration on of reduce sugar for determining the activity of enzyme.

EFFECT OF CARBON SOURCE

1ml fresh inoculum was taken which was concerned with the supplementation of carbon source (sucrose, Raffinose, lactose, glucose, starch, and mannose) at concentration (1%, 1%, 1%, 1%, 1%, and 1%) (W/w) level inoculated in to each 10 gram of substrate which was mixed up with mineral salt medium, after that let it allow for the incubation

at 30±1ºC for 72 hours at pH 7.0 at last the concentration of reducing sugar was formed by the hydrolysis of carboxyl methyl cellulose and by this phenomena enzyme activity was assayed to measured.

EFFECT OF NITROGEN SOURCE

1ml fresh inoculum was taken which was concerned with the supplementation of nitrogen source (Glycine, Peptone, Urea, Ammonium Chloride, Ammonium Sulphate, and Ammonium Nitrate) at concentration (1%, 1%, 1%, 1%, 1%, and 1%) (W/w) level inoculated in to each 10 gram of substrate which was mixed up with mineral salt medium, after that let it allow for the incubation at 30±1ºC for 72 hours at p^H 7.0 at last the concentration of reducing sugar was formed by the hydrolysis of carboxyl methyl cellulose and by this phenomena enzyme activity was assayed to measured.

PURIFICATION OF CRUDE ENZYME

After 72 hrs production, the enzyme was extracted and assayed as procedure given before. After that the extract was purified by the following techniques.

AMMONIUM SULPHATE PRECIPITATION

Ammonium sulphate gave 80% saturation at 4ºC by adding it in supernatant after that mixture kept as it was for overnight and resulting precipitation was collected as 8000rpm for 15 min**Bajaj *et al.,* (2009).**

DIALYSIS

Diffusion of solutes and ultra filtration of fluid across a semi permeable membrane were done by dialysis bag. In which the resulted precipitate was dissolved into the minimum volume of 0.01M phosphate buffer at pH 7.0 and it can re-further dialysis against same buffer for 24hrs at 4 ºC**Bajaj *et al.,*(2009).**.

ION-EXCHANGE CHROMATOGRAPHY

The dialyzed solution was applied with DEAE-Cellulose and the resultant column equilibrated with 0.01M phosphate buffer at pH 7.0 and again equilibration column was packed with the DEAE solution. So as the rate of flow was equal to 1ml/min. after that the sample was allowed to load by adding the buffer the step elution was done with sodium phosphate at pH7.0 from 0.1-1.0M NaCl gradient. At the last every eluted samples were collected to check cellulase activity and to determine each fraction by cellulase assay procedure ().**(Bajaj *et al.,* 2009).**.

PROTEIN ESTIMATION

The entire crude and purified enzyme's protein was estimated by using bovine serum albumin (BSA) taken as standard an extract by **Lowery *et al.,* (1951)**. Preparation of bovine serum albumin solution (BSA) different dilution can be done by mixing stock BSA solution (1mg/ml) after water into the test tube at given into the table 3.1 among these other test tube in which 2ml of alkaline copper sulphate reagent (analytical reagent) (App.) was added mixed well. After that solutions were incubated at room temperature for 10 min. again 0.2ml reagent Folin-Ciocalteau reagent solution(Lowery reagent solution) was added to each tube and incubated for 30 min. at last the absorbance was measured up to 550nm. To get standard calibration curve (Fig.) Absorbance's were plotted against protein concentration. The absorbance of unknown sample was determined using standard curve plotted below concentration. The absorbance of unknown sample was determined using standard curve plotted below**Lowry *et al.,*(1951).**

Table1: Lowry method for protein estimation

BSA (ml)	Water (ml)	Sample concentration (mg/ml)	Sample volume(ml)	Alk. $CuSo_4$ (ml)	Lowry reagent (ml)
0	5	0	0.2	2	0.3
1	4	0.1	0.2	2	0.3
2	3	0.2	0.2	2	0.3
3	2	0.3	0.2	2	0.3
4	1	0.4	0.2	2	0.3
5	0	0.5	0.2	2	0.3

Table depicts about the protein estimation

Characterization of modified cellulase

pH optimum

The effect of different incubation pH (4-9) on enzyme activity was being determined using two buffer solutions. 0.01M phosphate, pH 4.0, 4.5, 5.0, 5.5, 6.0; 6.5, 7.0, 7.5, 8.0, 8.5 and pH 9.0**Kim,C.H (1995)**.normal enzyme assay was performed after 15 minutes of incubation using CMC as substrate and results observed at a 550nm**Beg and Gupta (2003)**.

Temperature optimum

The effect of different incubation temperatures were (25-60ºC) on the purified enzyme. The purified

cellulase was incubated under different temperatures conditions **Fadal, M. (2000)**. After 15 minutes of incubation cellulase was assayed to determine the effect of temperature on enzyme activity with the same procedure **Haddar *et al.*,(2009).**

Industrial Application

Detergent compatibility of cellulase

Four locally available detergent brands were be used for studying compatibility of purified carboxymethyl cellulase (CMC) under normal conditions. Detergent solutions were being prepared as per directions given on their respective sache. Carboxymethyl cellulase solution (1%) was be used as substrate and prepared in 0.01M phosphate buffer of pH 7.0. A reaction mixture comprising 3ml of substrate solution, 1.1ml detergent solution and 0.9ml purified cellulase will be incubated at room temperature for 10-15 minutes followed by normal enzyme assay as described. A control sample will also be incubated in parallel to reaction mixture solution.

STASTICAL ANALYSIS

χ^2 -Test

$$\chi^2 = \frac{(Oi - Ei)^2}{Ei}$$

$$E_i = \frac{\text{Sum of all frequencies}}{N}$$

Where,

O_i = Observed frequency in each category

E_i= Expected frequency in each category

d.f. = Degree of freedom (n-1)

χ^2= Chi square

$\chi^2_{(tab)}$= 14.06 at 5% level

n = Number of Sample

Analysis of variances table (ANOVA):-

Table 2: Analysis of variances table (ANOVA)

Source of variation	Degree of freedom	Sum of square	Mean sum of square	F cal.	F tab (5%)	Result
Due to replication	(r-1)	R.S.S.	$\frac{R.S.S.}{(r-1)}$	$\frac{M.S.S.R.}{M.E.S.S.}$	F(r-1).(r-1)(t-1)	S/NS
Due to treatment	(t-1)	T.S.S.	$\frac{T.S.S}{(t-1)}$	$\frac{M.T.S.S.}{M.E.S.S}$	F(t-1).(r 1)(t-1)	S/NS
Due to error	(r-1)(t-1)	E.S.S.	$\frac{E.S.S.}{(r-1)(t-1)}$	-	-	-
Total	(rt-1)	TSS	-	-	-	-

IF $F_{(Cal)} > F_{(Tab)}$ = Significant, IF $F_{(Cal)} < F_{(Tab)}$ = Non Significant

S.E (d) for Treatment = $\sqrt{\frac{2 \times MESS}{r}}$

C.D. at 5% = S.E.D × $t_{(5\%)}$ error d.f.

Where,

d.f. = Degree of freedom
S.S. = Sum of square
M.S.S. = Mean sum of square
F (cal.) = Calculated value of 'F'
F (tab.) = Table value of 'F'
R.S.S. = Sum of square to replicate
E.S.S = Error sum of square
T.S.S. = Total sum of square
M.R.S.S = Mean sum of square (Replication)
S.S.T. = Sum of square due to treatment
M.E.S.S. = Mean sum of square due to error
M.S.S.T. = Mean sum of square (Treatment)

Standard error due to Mean –

Standard error of mean was calculated by the following formula-

$$\text{S.E.M.} = \frac{\sqrt{EMSS}}{r}$$

Where-

E.M.S. = Error mean sum of square
F = Number of replication
T value = Table value at error degree of freedom at 5% level of significant.

Test of significant-

If the variance ratio (or) f- calculated value (MST/MSV) or treatment was greater than the F-table value at 5% and 1% level of signification the variance between treatments was considered to be significant. If the calculated value is less than the F tableted value the difference between treatments were considered to be no significant.

$$t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$$

If $t_{(cal)} > t_{(tab)}$ =significant

$t_{(tab)}$ = at 5% level
$t_{(tab)}$ = at 5% level
$t_{(tab)}$ = at 5% level

III. RESULTS &DISCUSSION:

The aim of present study was to "production, optimization, partial purification and application of bacterial cellulase from agro-waste". The culture conditions were optimized to achieve highest cellulase activity and afterwards, produce cellulase was subjected to purification, characterization and industrial application.

ISOLATON

A total 100 soil sample were collected from different place of Allahabad such as Garden soil, wood furnishing regions, sugarcane farm soil, petrol pump, chimney regions. Incidences of cellulolytic bacteria were detected from all the places except chimney and petrol pumps. Maximum incidences (55%) of cellulolytic bacteria were found to be present in garden soil and sugar cane farm soil sample.

The incidence of cellulolytic bacteria from different site of Allahabad was different due to many reasons like soil environment, season and time of collection of soil samples. The incidence of cellulolytic bacteria reported by **Wang *et al.*,(2008)** is in agreement with the present study. Cellulolytic bacteria observed in the present study from soil sample are comparable with study of **Das and Murali(2010)**.

Table :3 Incidence of cellulolytic bacteria from different source

Total Samples	No. of Bacillus Spp.	Incidence (%)				
		Garden Soil Sample	Wood furnishing	Sugar Cane	Petrol pump	Chimney region
100	40 (40%)	17 (42.5)	9 (22.5)	14 (35)	0 (0)	0 (0)

$\chi^2_{(Cal)} = 30.75 > \chi^2_{(Tab)} = 14.067$ at 5% level (Significant)

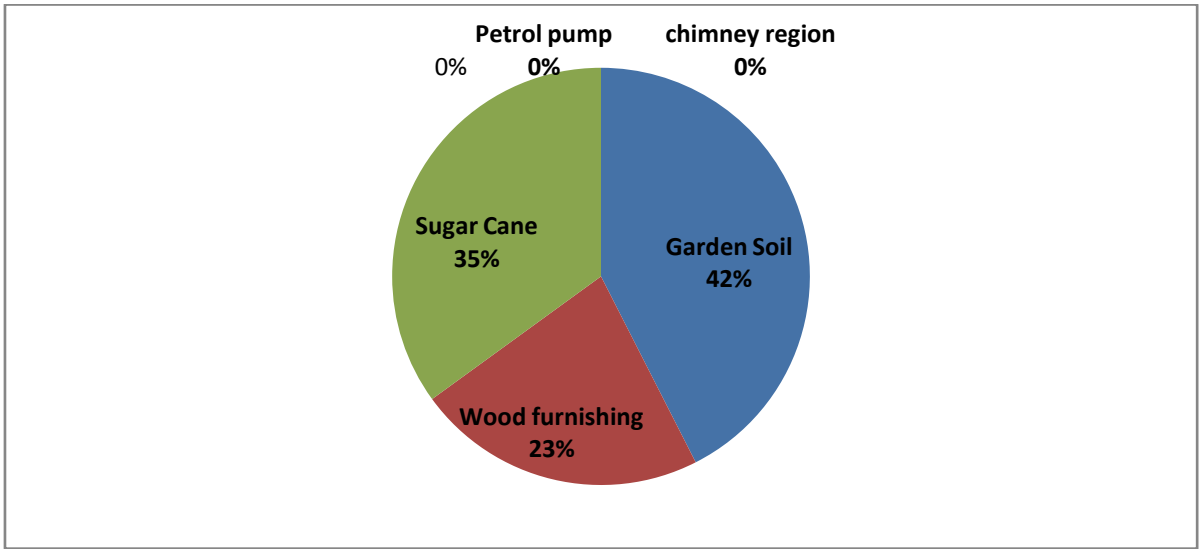

Fig 1: incidence of cellulolytic bacteria from different source.

Identification

The strain showing maximum cellulase activity within short period of time was selected for further studies. Then the selected strain were identified on the basis of cultural, morphological and biochemical characteristics . The bacterial culture were found creamy, circular, entire flate and long rod shape, gram positive in cultural and morphological characteristics. Bacterial culture showed all positive results in catalase test. In the starch hydrolysis test, gelatin test and all other biochemical tests bacterial culture showed some positive and some negative results.

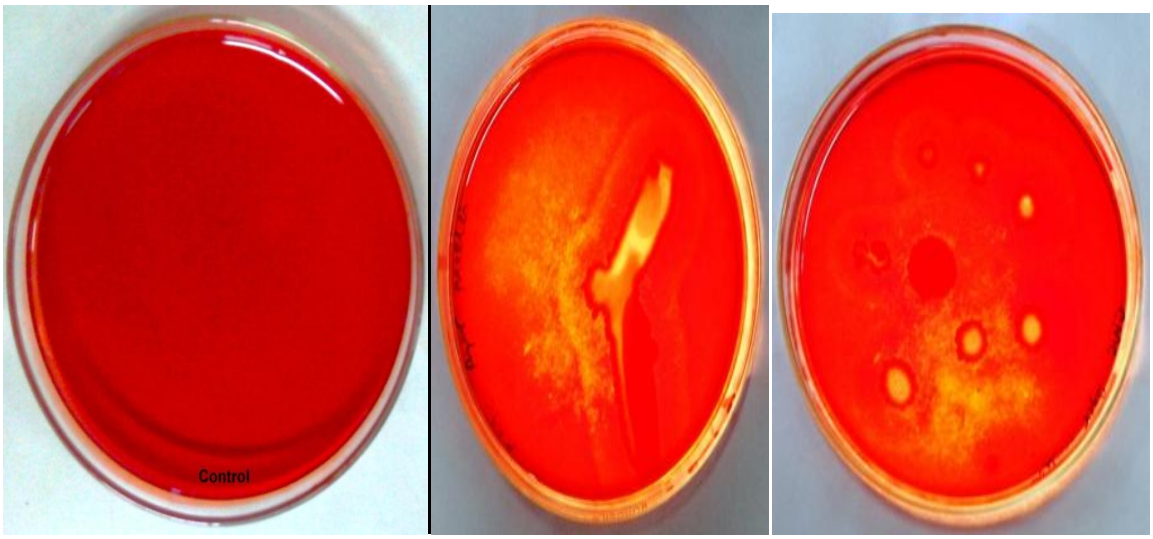

Fig 2.0 The formation of clear zone of hydrolysis indicated cellulose degradation.

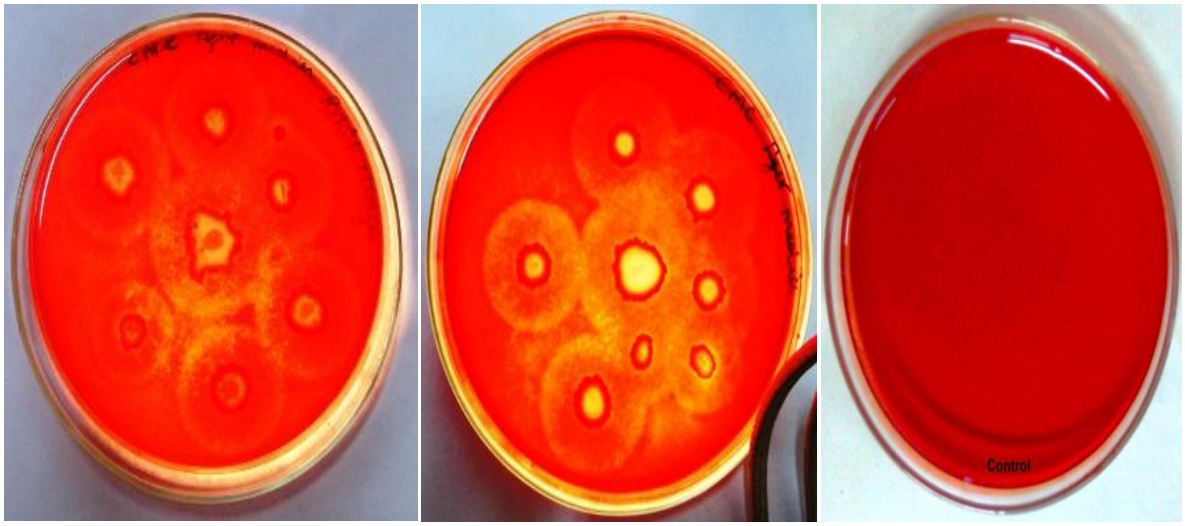

Fig 3 The formation of clear zone of hydrolysis indicated cellulose degradation.

Fig 4Quadrant streak of *Bacillus Spp.* Bacteria on Nutrient Agar (NA) medium

Fig 5.Zig-zag streak of *Bacillus Spp.* bacteria on nutrient agar (NA) medium

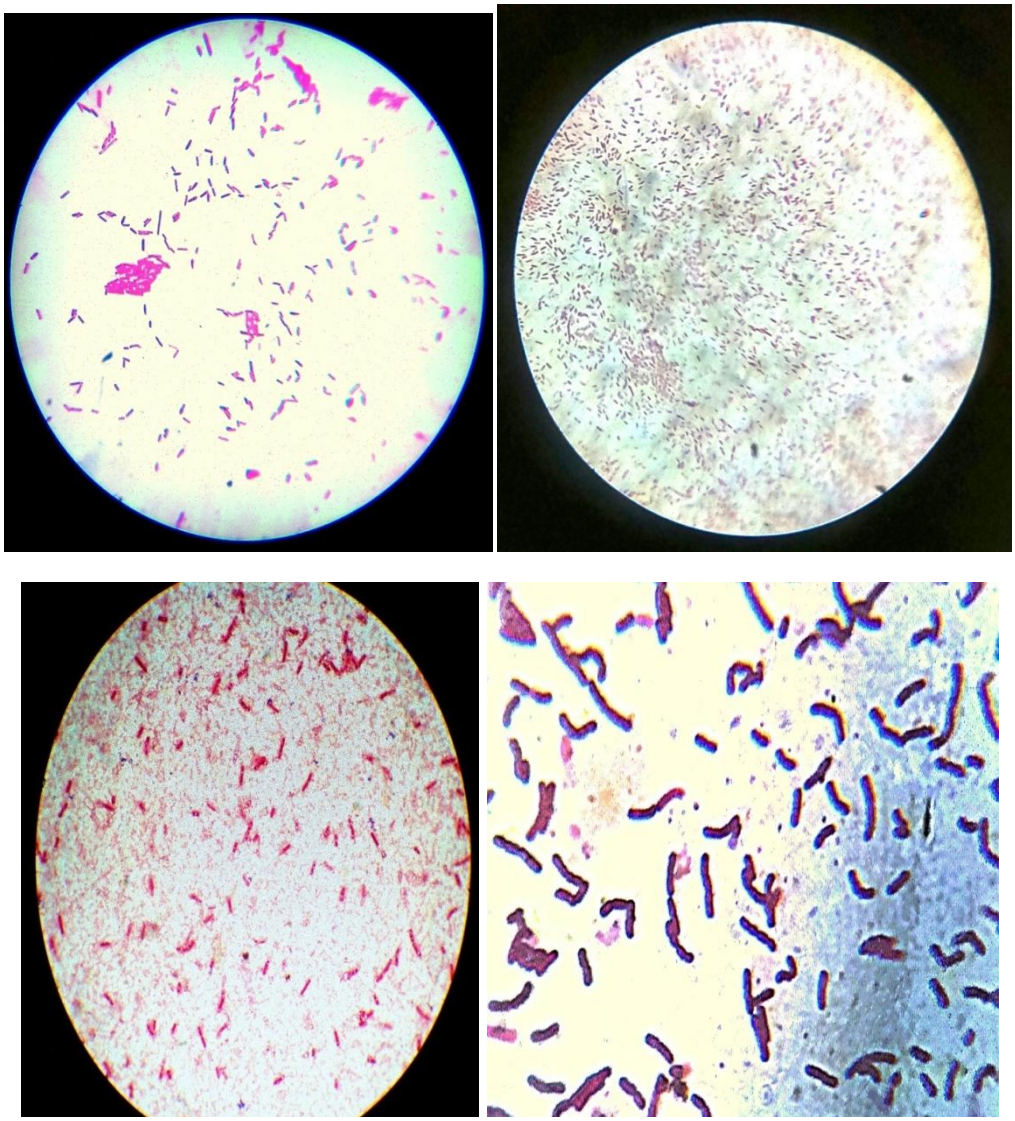

Fig 6: Microscopic Observations of *Bacillus spp.* bacteria at 100X

Table5: Identification of Isolated Bacteria by Cultural and Morphological Characteristic

Isolate	Colony colour	Margin	Elevation	Shape of colony	Gram stain reaction	Cell shape	Organism
B.Z(A)	White	Irregular	convex	Flat	+ve	Rod	*Bacillus cereus*
B.Z(B)	Off white	Irregular	convex	Round	+ve	Rod	*Bacillus Pantothenticum*
B.Z(C)	White	Irregular	convex	Round	+ve	Rod	*Bacillus Insolitus*
B.Z(D)	Creamy white	Regular	convex	Round	+ve	Rod	*Bacillus Insolitus*
B.Z(E)	Yellowish white	Irregular	concave	Round	+ve	Short Rod	*Bacillus alvei*
B.Z(F)	Creamy	regular	concave	Circular	+ve	Rod	*Bacillus Firmus*
CA	Yellowish white	Irregular	convex	Round	+ve	Rod	*Bacillus alvei*
C9	Off white	Irregular	convex	Round	+ve	Rod	*Bacillus alcolphilus*
C10	White	Irregular	concave	Round	+ve	Rod	*Bacillus alvei*
C17	Creamy white	Irregular	convex	Circular	+ve	Rod	*Bacillus subtilus*

Table.6 Biochemical Tests

BACILLUS SPP. / Biochemical Tests	B.Z(A)	B.Z(B)	B.Z(C)	B.Z(D)	B.Z(E)	B.Z(F)	CA	C9	C10	C17
Catalase Test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Methyl Red Test	-ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve
Voges-Proskauer Test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Citrate Utilization Test	+ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve
Indole-Formation Test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Gelatin Hydrolysis Test	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve
Starch HydrolysisTest	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Oxidase Test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Litmus Milk Test	Acid	Alkali	Alkali	Alkali	Alkali	Alkali	Alkali	Alkali	Acid	Alkali
Urease Test	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Motility Test	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
Triple Sugar Iron AgarTest	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
H_2S Production Test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Nitrate Reduction Test	-ve/G$^+$	-ve/G$^+$	-ve/G$^+$	-ve/G$^-$	-ve/G$^-$	+ve/G$^-$	-ve/G$^-$	-ve/G$^-$	-ve/G$^-$	-ve/G$^-$
Organism	*Bacillus Cereus*	*Bacillus Pantothenticum*	*Bacillus Insolitus*	*Bacillus Insolitus*	*Bacillus Alvei*	*Bacillus Firmus*	*Bacillus Alvei*	*Bacillus Alcolphilus*	*Bacillus Alvei*	*Bacillus Subtilus*

Table 7:SUGAR FERMENTATION TEST

Carbohydrate fermentation (Acid production - A, Gas production - G)

Bacillus Spp. / **Sugars Name**	B.Z(A)	B.Z(B)	B.Z(C)	B.Z(D)	B.Z(E)	B.Z(F)	CA	C9	C10	C17
D-Mannitol	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-
D-Glucose	A+/G-	A+/G-	A-/G-	A+/G-	A-/G-	A+/G-	A+/G-	A+/G-	A+/G-	A-/G-
D-Xylose	A-/G-	A-/G-	A+/G-	A-/G-	A-/G-	A-/G-	A+/G-	A-/G-	A-/G-	A-/G-
L-Arabinose	A-/G-	A-/G-	A-/G-	A-/G-	A+/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-
Lactose	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A+/G-	A-/G-	A-/G-
Sucrose	A+/G-	A+/G-	A-/G-	A+/G-	A+/G-	A+/G-	A-/G-	A+/G-	A+/G-	A-/G-
Galactose	A-/G-	A-/G-	A+/G-	A-/G-	A-/G-	A-/G-	A-/G-	A+/G-	A-/G-	A-/G-
Sorbitol	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-
Fructose	A+/G-	A+/G-	A-/G-	A+/G-	A+/G-	A+/G-	A-/G-	A+/G-	A+/G-	A-/G-
Rhannose	A-/G-	A-/G-	A+/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-	A-/G-
Trehalose	A+/G-	A-/G-	A-/G-	A-/G-	A-/G-	A+/G-	A-/G-	A+/G-	A+/G-	A-/G-
Organism	*Bacillus Cereus*	*Bacillus Pantothenticum*	*Bacillus Insolitus*	*Bacillus Insolitus*	*Bacillus Alvei*	*Bacillus Firmus*	*Bacillus Alvei*	*Bacillus Alcolphilus*	*Bacillus Alvei*	*Bacillus Subtilus*

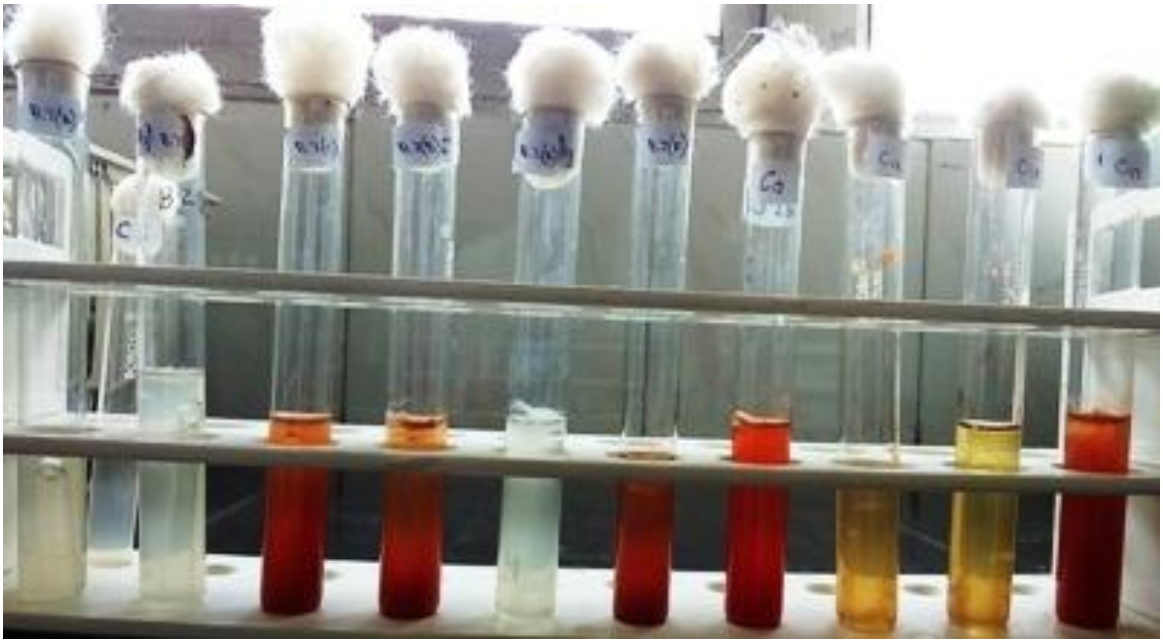

Fig:7- MR-VP test of *Bacillus Spp.* bacteria

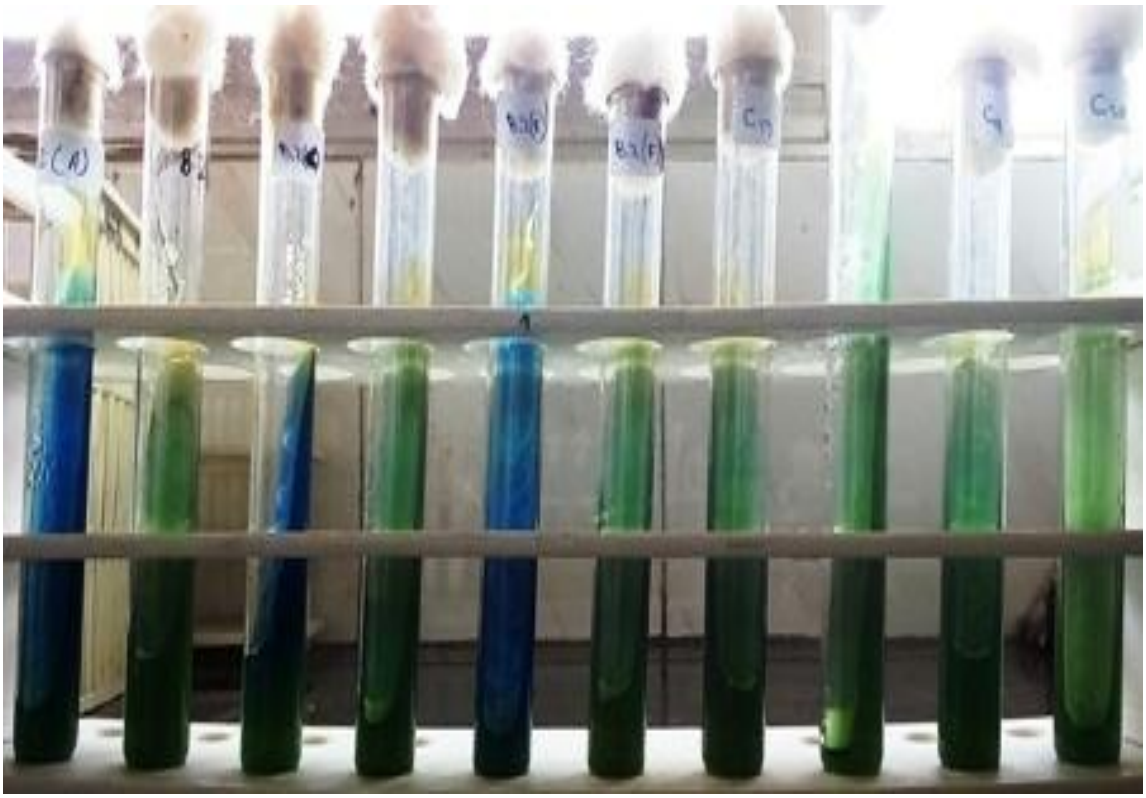

Fig-8: Citrate Utilization test of *Bacillus spp.* bacteria

Fig:9 Indole-Formation Test of *Bacillus spp.* bacteria

Fig: 10 Gelatin Hydrolysis Test of *Bacillus spp.* bacteria

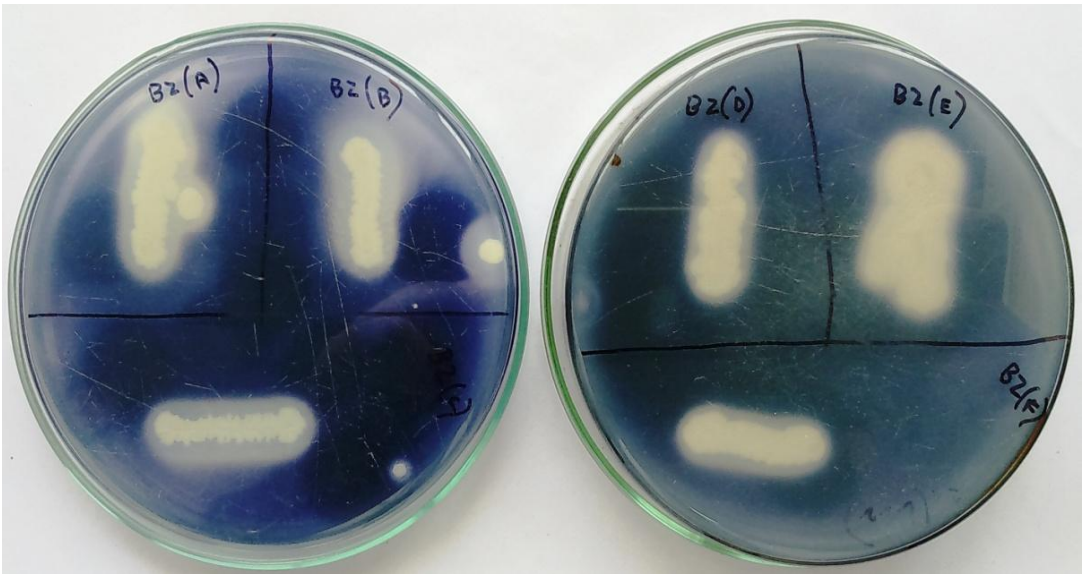

Fig 11 Starch Hydrolysis Test of *Bacillus spp.* Bacteria

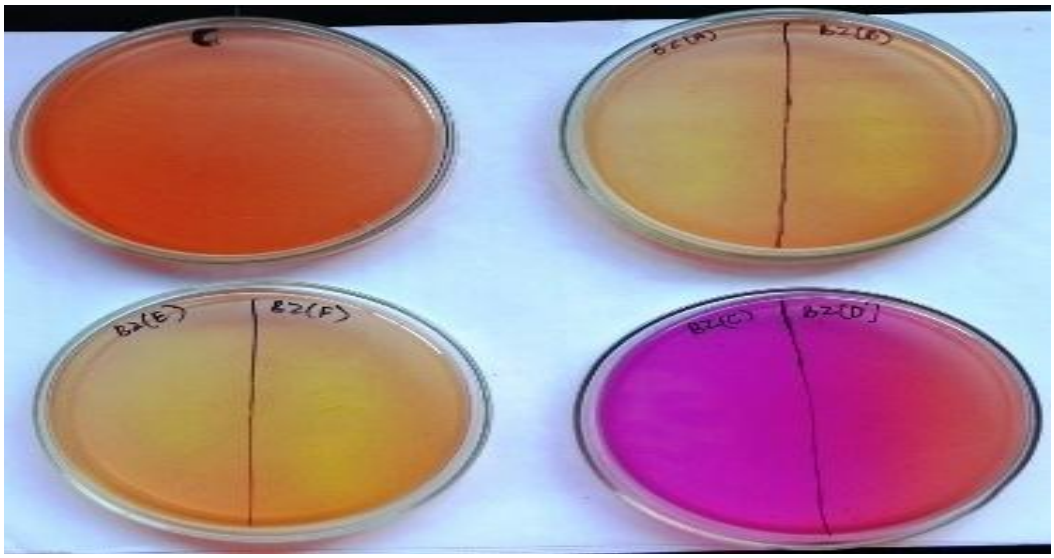

Fig 12: Urease Test of *Bacillus spp.* bacteria

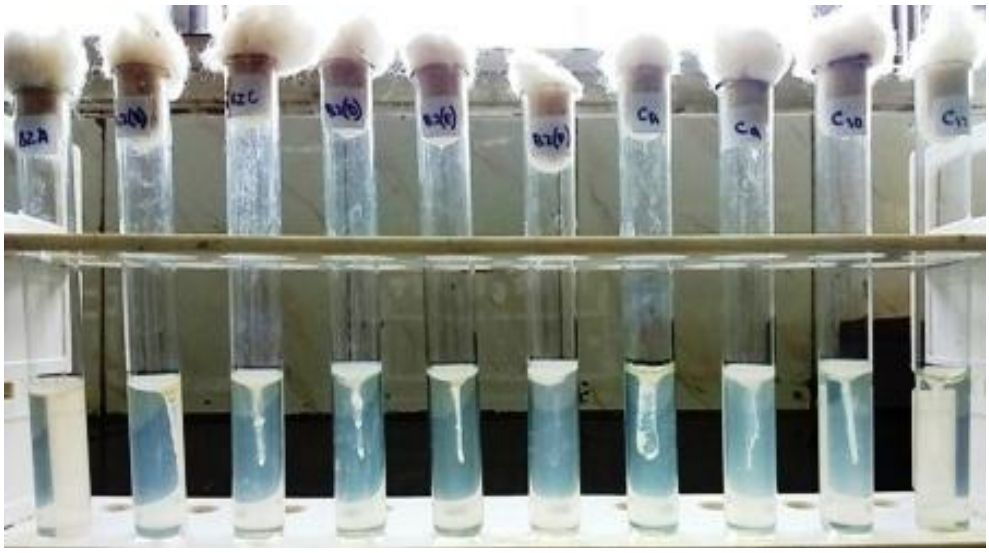

Fig 13:Motility Test of *Bacillus spp.* Bacteria

Fig 14: Triple Sugar Iron Test of *Bacillus Spp.* Bacteria

Fig 15: Nitrate Reduction Test of *Bacillus Spp.* bacteria

Fig 16: Litmus Milk Test of *Bacillus Spp.* bacteria

Fig17 Sugar Fermentation Test of Bacillus Spp. bacteria

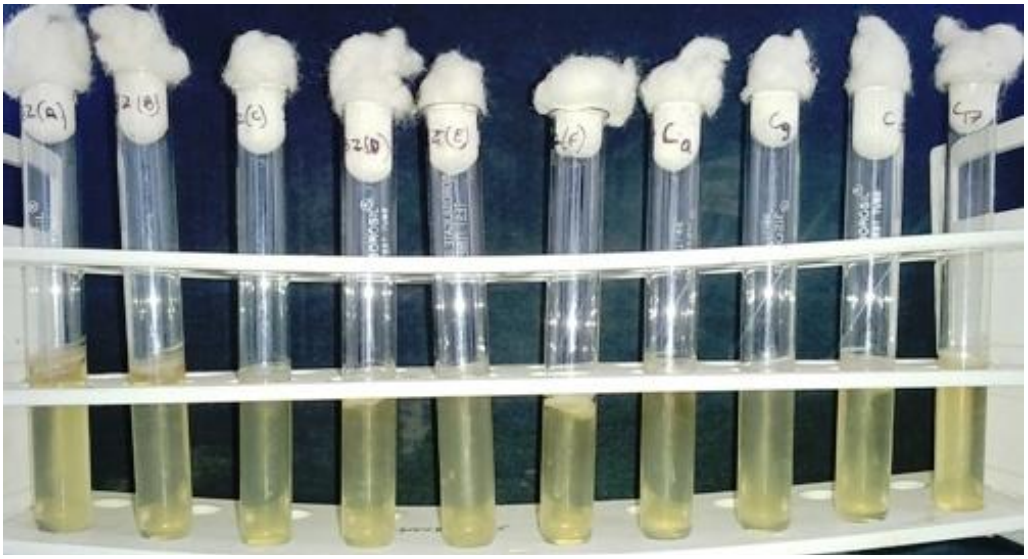

Fig 18: H_2S Production Test of Bacillus Spp.

SELECTION OF BEST SUBSTRATE

In the study substrate sugar cane Bagasse, Chana husk, wheat straw, ground nut cell, Rice straw were used. Among all the substrate sugar cane Bagasse gave the maximum cellulase production (avg.75.42 U/ml) on 3rd day when fermented with *Bacillus Spp.* bacteria under SSF (table 4.3; Fig. 4.2). Rice straw was found as a poor substrate among others.

The total enzyme titres were 5-7-folds higher in sugar cane Bagasse than those when sugar cane stem, Chana husk, wheat straw, ground nut cell and rice straw were used as substrate. Total cellulase production by sugar cane Bagasse was more. There results support the suitability of using sugar cane Bagasse as solid substrate for high production of cellulase **Iqbal *et. al.*,(2011)**. The data were analyzed statistically using f-statistics and the result showed significant result at 5% probability level (App IV-3.1).

Table 8: Selection of best substrate

Enzyme activity (U/ml) at different hrs.						
S.No	Substrates	24hrs	48hrs	**72hrs**	96hrs	120hrs
1	Sugar cane Bagasse	0.11	0.273	**0.383**	0.283	0.173
2	Chana Husk	0.019	0.039	0.171	0.11	0.09
3	Wheat Straw	0.017	0.024	0.041	0.031	0.029
4	Ground Nut Cell	0.015	0.021	0.037	0.031	0.029
5	Rice Straw	0.005	0.009	0.011	0.009	0.007

Due to Substrate: $F_{(Cal)} = 10.67 > F_{(Tab)} = 2.26$ (Significant) at 5% level
Due to Incubation period: $F_{(Cal)} = 1.90 < F_{(Tab)} = 2.26$ (Non-Significant) at 5% level

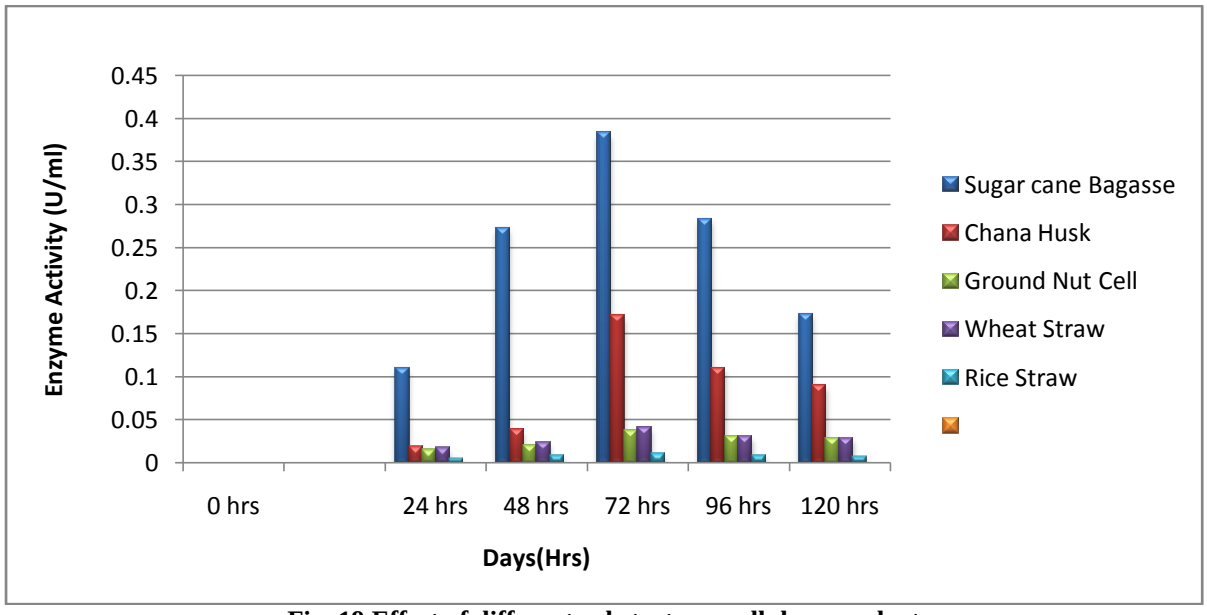

Fig. 19.Effect of different substrate on cellulase product

OPTIMIZATION OF MEDIA PARAMETER FOR MAXIMUM CELLULASE PRODUCTION FROM BACILLUS SPP.

EFFECT OF INCUBATION PERIOD

During the course of study, the activity of bacillus spp. bacteria was detected in the production medium Supernatant since the first day to fifth day. The major peak of activity was found after 48hrs. Incubation beyond the optimum time showed a rapid decline in the enzyme yield, as compare to maximum (avg. 16.45U/ml) at 72hrs. The data were analyzed statistically using t-statistics, line regression, and the result showed non- significant result at 5% probability level.

The reduction in cellulase yield after an optimum period is probably due to depletion of

nutrient available to micro-organisms. A maximum of 72hrs was reported for the optimal cellulase production by *Bacillus Spp.* bacteria **wanderley *et al.*,(2004). Dees *et al.*,(2002)** and **Heck *et al.*,(2002)** reported that *Bacillus Spp. B2*, *Bacillus pumllis* and *Bacillus subtilis* shows maximum cellulase activity at 72hrs.of incubation period. **Shafique *et al.*,(2004)** also reported72 hrs by bacillus subtilis by using sugar cane Bagasse as solid substrate. However, this was in contrast with the finding of many other workers, whom recorded maximum cellulase productivity after 36 hrs. By *Arachnoitus Spp.***Ahmed *et al.*,(2004)**, 120hrs. By *Bacillus Pumilus***Poorna and prema, (2007)** and 142hrs. Incubation by *Clostridium Cellulolyticum***Guedeon *et al.*,(2002).**

Table 9: Enzyme Activity of cellulase from Bacillus cereus. Bacteria at different incubation period.

S. No.	1	2	3	**4**	5	6
Incubation Period (Hrs)	0	24	48	**72**	96	120
Enzyme Activity (U/ml)	0	1.04	4.37	**16.45**	9.45	5.6

r = 0.38, $t_{(cal)}$ = 0.71< $t_{(tab)}$ = 3.18at 5% level (Non Significant) y = 1.763x + 0.332

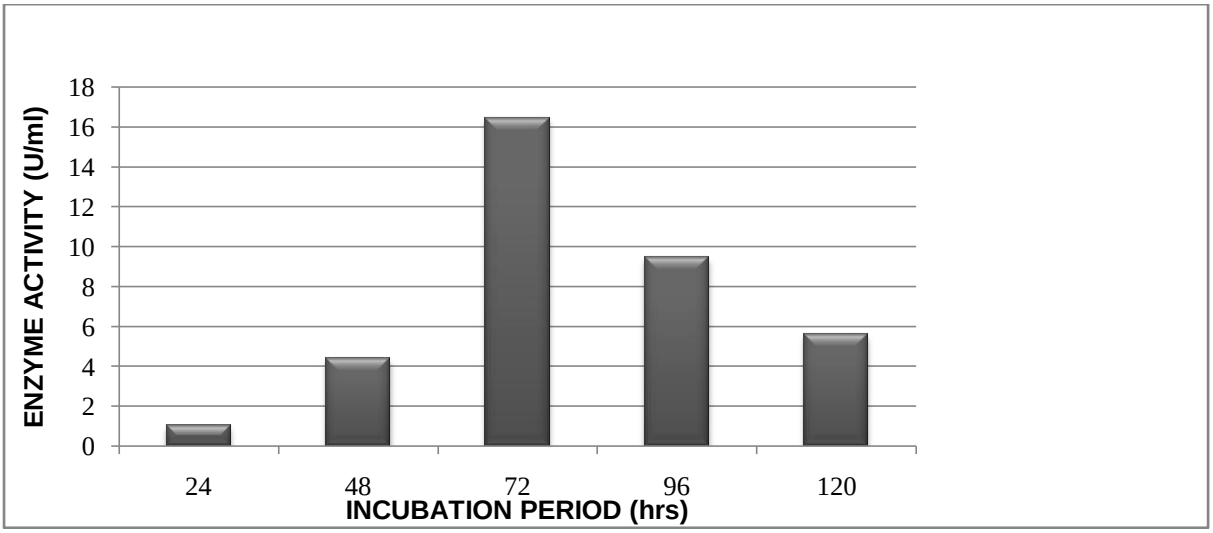

Fig 20: Production of Cellulase from Isolated Bacillus cereus. at different Incubation Period.

Effect of Incubation Temperature

The data obtained during the course of time of study indicated that there is a significant effect of temperature on cellulase production. Beyond the optimum temperature 30°C; a sharp fall in cellulase activity was obtained. The cellulase activity was minimum **(5.55U/ml)** at **25°C** and maximum **(43.09U/ml)** at **30°C**. The data were analyzed statistically using t-statistically using t-statistics, line regression and the result showed non-significant result at 5% probability level.

The Optimum temperature of 30°C was found to maximally influence the *Bacillus* Spp. to produce the cellulase. Similarly **Kotchoni *et al.*,(2003)** and **Poorna and prema, (2007)** reported that mutant *Bacillus pumilus* and *Cellulomonas* spp. shows maximum cellulase activity at 30°C. However, these results were in contrast with the data recorded by many other workers such as **Amritkar *et al.*, (2004), Krishna and Chandrasekaran, (1999), Shafique *et al.*,(2004)** observed optimum temperature of 35°C for *Bacillus Subtilis* CBTK106 and *Bacillus Spp.B21.* and 37°C for *Bacillus* Spp. *Clostridium cellulolyticum* Cel19M and *Pseudomonas fluorescens***Bakare *et al.*, (2005); Heck *et al.*, (2004)**.

Table 10:Enzyme activity of cellulase from Bacillus cereus bacteria at different incubation temperature

S.No	1	2	3	4	5
Temperature (°C)	25	**30**	37	40	50
Enzyme activity (U/ml)	5.55	**43.09**	31.2	19.65	12.85

r = 0.13, $t_{(cal)}$ = 0.013< $t_{(tab)}$ = 3.18 at 5% level (Non Significant) y = 1.763x + 0.332

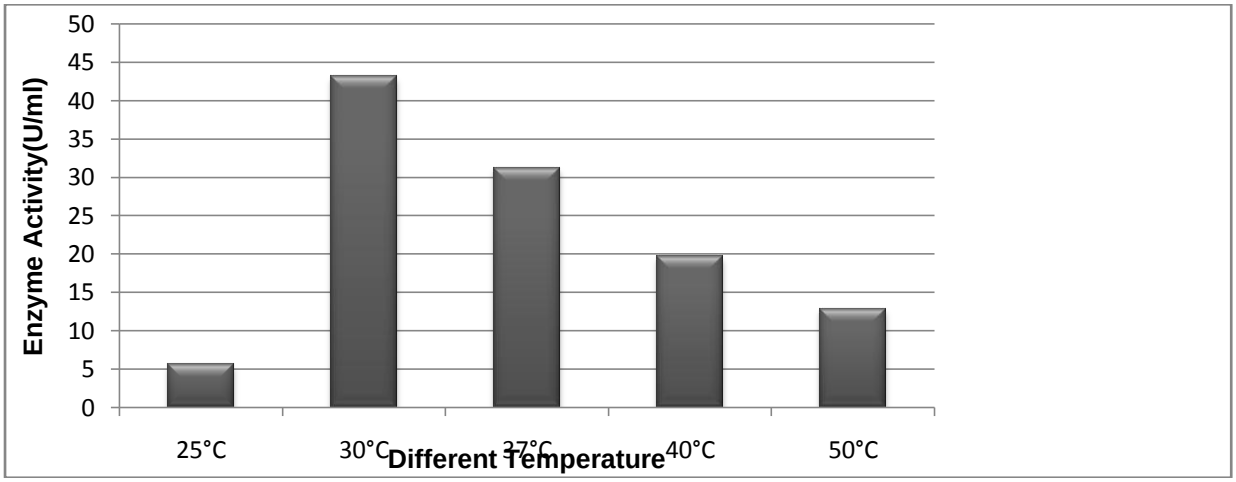

Fig 21 :Production of cellulase from Bacillus cereus bacteria at different incubation temperature

EFFECT OF INITIAL pH

The data obtained indicate there is strong influence of pH on cellulase production. The enzyme activity declines on either side of the optimum pH value. In present study the optimum pH 7.5 (14.59U/ml) was maximum reported for cellulase production from isolated culture. The minimum cellulase production was at pH 4.0 (0.38U/ml) Table (4.6; fig 4.5). The data were analyzed statistically using t-statistics, line regression and the result showed non-significant result at 5% probability level.

Similarly maximum cellulase productivity was found at pH 7.5 by *B. circulance* **Ray *et al.*,(2007).** Many earlier reports reported maximum cellulase productivity at pH 7.0, for example *Lysobacter spp.***Ogura *et al.*,(2006),***Cellulomonas*, *Bacillus* and *Micrococcus spp.* **Immanuel et al.,(2006)**. Maximum cellulase productivity was found to be 5.0-6.5 in bacillus strain **Mawadza *et al.*,(2000).***Bacillus licheniformis* cellulase was found to be more stable under acidic condition **Bischoff *et al*.,(2006).**

Table 10: Enzyme activity of cellulase from Bacillus cereus bacteria at different pH

S.No.	1	2	3	4	5	6	7	8	9	10	11
pH	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
Enzyme activity (U/ml)	0.38	2.09	2.97	3.49	4.52	11.31	12.21	14.69	5.79	5.422	4.02

r =0.45, $t_{(cal)}$ = 1.55 < $t_{(tab)}$ = 2.26 at 5% level (Non Significant) y = 1.763x + 0.332

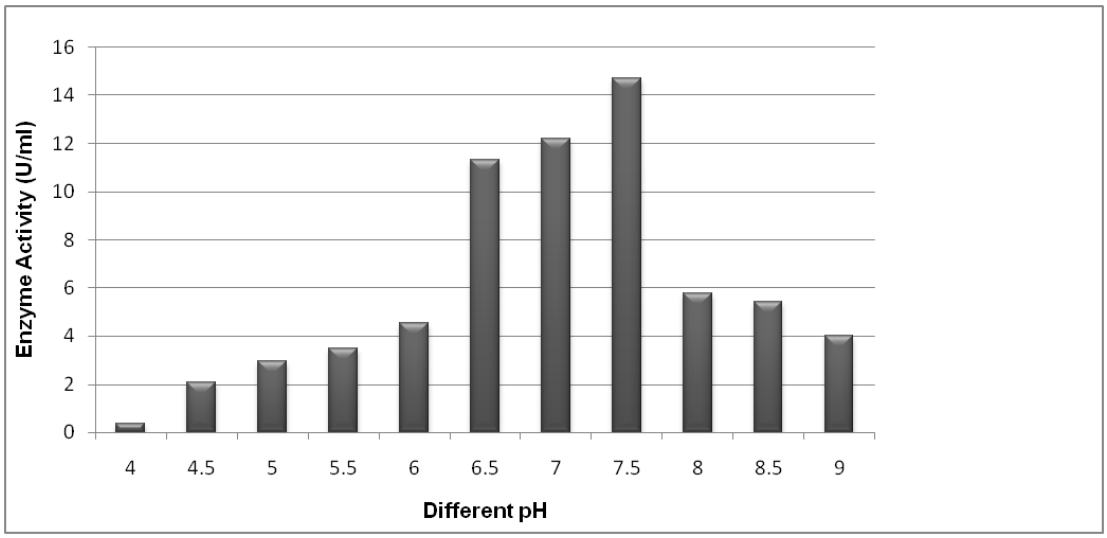

Fig.4.6 Production of cellulase from Bacillus cereus bacteria at different pH

EFFECT OF CARBON SOURCE

The enzyme activity was studied for different carbon source at same concentration of 1%w/v. Among these Carbon sources lactose produced maximum cellulase activity as compared to glucose. The maximum activity was showed at 1% of lactose (30.63U/ml) and minimum at 1% of glucose (14.7U/ml) . The data were analyzed statistically using f-statistics and the result showed significant result at 5% probability level. (App IV-3.2).

Similar findings were also reported by **Bai *et al*.,(2012)** where they found lactose as the best carbon source for maximum production of Cellulase of 23.96 U/ml. in a similar study done by **Jaradat et al., (2008)** glucose has been found to be the best carbon source for cellulase production by *Streptomyces* sp. Utilization of CMC as carbon source is best for cellulase production as reported by **Das et al., (2010)** for *Bacillus* sp (3.028 µg/mg/min). Production of cellulase was enhanced by the additional carbon sources like Sucrose, Raffinose, Lactose, Mannose, Starch and glucose in Bacillus Sp. **Teodoro *et al*.,(2000).**

Table :11 Enzyme activity of cellulase from Bacillus cereus bacteria at different carbon sources

S.No.	1	2	3	4	5	6
Name of Carbon Sources	Sucrose	Raffinose	Lactose	Mannose	Starch	Glucose
Enzyme Activity(U/ml)	22.92	19.93	30.63	24.31	16.27	14.7

Due to Substrate: $F_{(Cal)} = 27.87 > F_{(Tab)} = 2.22$ (Significant) at 5% level
Due to Incubation period: $F_{(Cal)} = 5.90 > F_{(Tab)} = 2.22$ (Significant) at 5% level

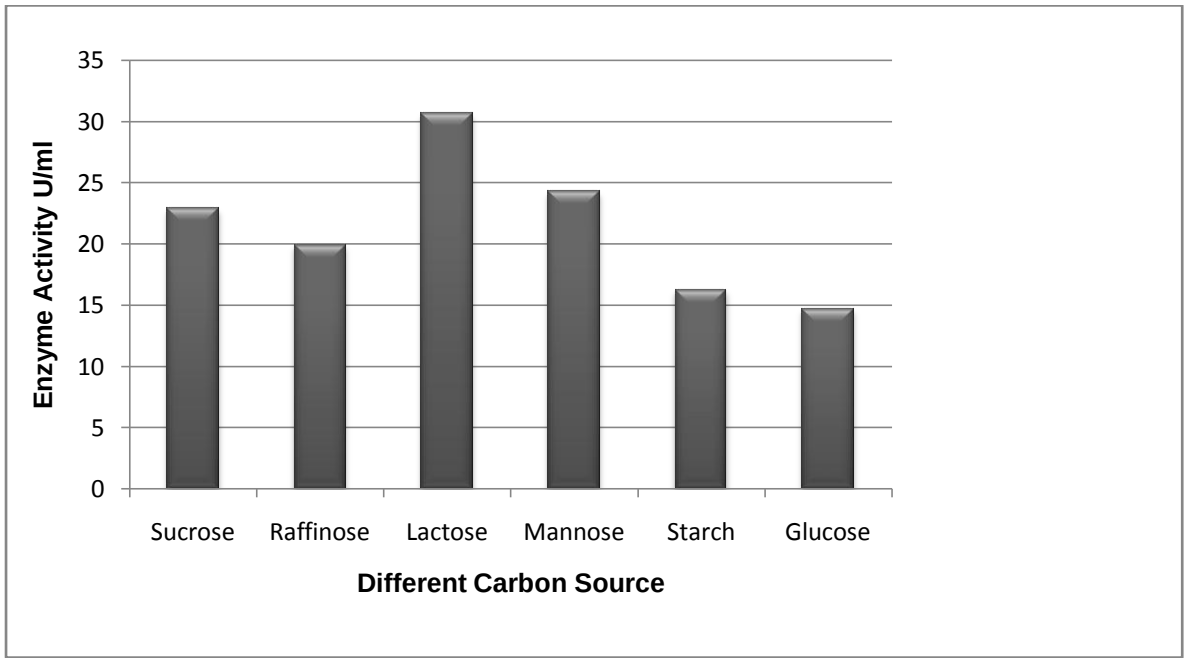

Fig.23: Production of cellulase from *Bacillus* cereus bacteria at different carbon source.

EFFECT OF NITROGEN SOURCE

The enzyme activity was studied for different nitrogen sources at same concentration of 1%W/v. among these nitrogen sources peptone produced maximum cellulase activity as compared to urea. The maximum activity was showed at peptone **(19.95U/ml)** and minimum activity was at glycine **(1.2U/ml)**. The data analyzed statistically using f-statistics and the result showed significant result at 5% probability level. (App IV-3.3).

Findings of the present study are in accordance with **Doi (2008)** who found peptone as best nitrogen source for cellulase production. Similarly **Das *et al*.,(2010)** found peptone as best nitrogen source for cellulase production.Peptone in the medium resulted in high cellulase activity in the culture supernatant which is calculated as 0.474 IU/ml/min at 35˚C for 48 hours of incubation **Irfan et al.,(2012)**. Peptone was found to be the better nitrogen source as it increases the production of Cellulase up to 29.63 U/ml **Bai et al.,(2012).** However, results of present study were in contrast with **Jaradat *et al*.,(2008)** who achieved maximum cellulase production in a medium containing NH_4Cl as a nitrogen source.

Table 11: Enzyme activity of cellulase from *Bacillus* cereus bacteria at different nitrogen sources

S.No.	1	2	3	4	5	6
Name of nitrogen source	Glycine	Ammonium sulphate	Ammonium Chloride	Peptone	Urea	Ammonium Nitrate
Enzyme activity(U/ml)	1.2	3.49	2.07	19.95	12.95	11.72

Due to Substrate: $F_{(Cal)} = 129.94 > F_{(Tab)} = 2.22$ (Significant) at 5% level
Due to Incubation period: $F_{(Cal)} = 3.67 < F_{(Tab)} = 2.22$ (Significant) at 5% level

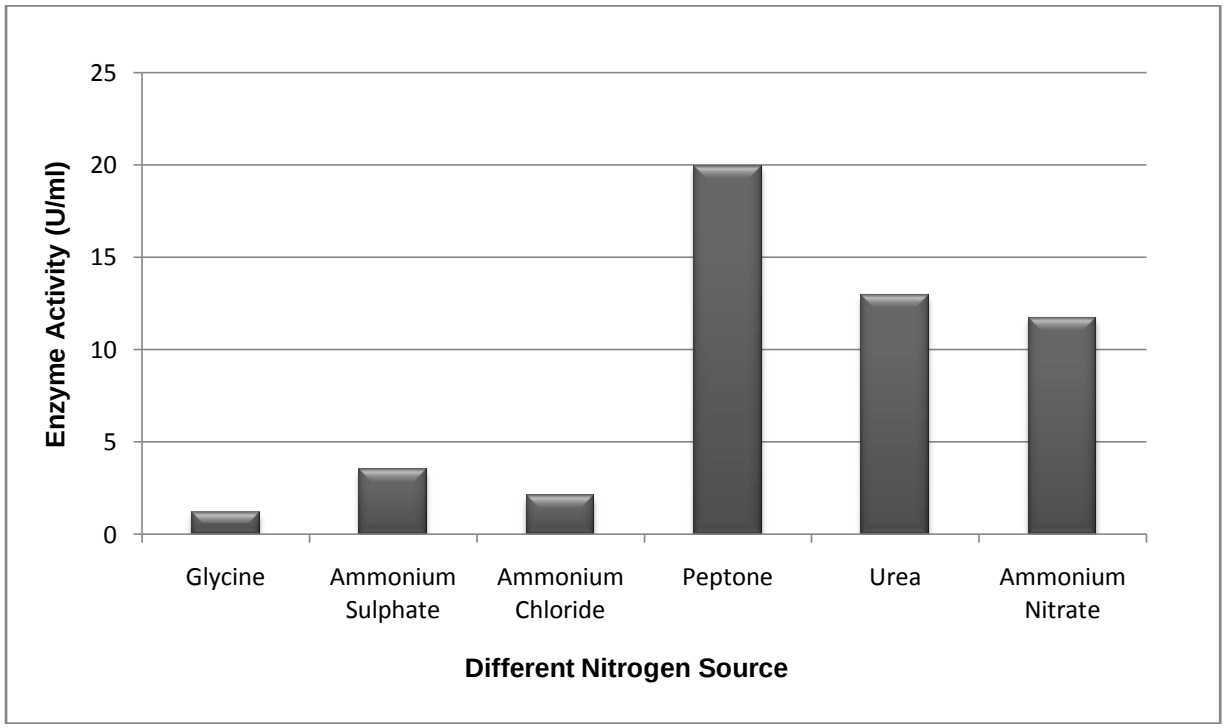

Fig.24. Production of cellulase from *Bacillus*cereus bacteria at different nitrogen source

PRODUCTION OF ENZYME IN BEST OPTIMIZED CONDITIONS

The mineral salt solution was taken according to the optimized parameter after optimization. It had the best optimized conditions-:

- Incubation period :- 72 hours
- Initial pH :- 7.5
- Incubation Temperature :- 30 ºC
- Additional Carbon Source :- 1% Lactose
- Additional Nitrogen Source :- 1% Peptone

CELLULASE PARTIAL PURIFICATION AND PROTEIN DETERMINATION

The maximum yielding condition the cellulase was produced by *Bacillus Spp.*Culture. The crude enzyme showed the enzyme activity of 1.77 U/ml. the crude waspurified by precipitating it with different concentration of solid ammonium sulphate (40%,60% and 80% saturation)**Lee *et al.*, (2006).** The mixture was left overnight at 4ºCIn a magnetic stirrer. There was observed that 80%concentration of ammonium sulphateshowed better performance for the enzyme precipitation. The recovery was furtherfollowing by dialysis and ion exchange column chromatography yielding the highestactivity **Bajaj *et al*,.(2009).** the elution was done with gradient ranging from 0.01-1MNaCl and the highest activity were yielded with 0.01M NaCl Concentration.

Protein of both crude and purified enzyme was estimated by using bovine Serum albumin as standard **Lowry *et al.*,(1951)**. The yield was gradually decreases in every Purification steps. the purified crude enzyme had 75.62mg/ml where as the protein had 1633.17mg/ml. DEAE-cellulase chromatography resulted in purification fold of 1.50 **De-Moraes *et al.*,(1999)** reported in bacillus strain M-9 that DEAE-cellulase

Chromatography resulted in purification fold of 3.47 to 9.06. Sinorhizobium fredii cellulose was purified by 9.08 folds using ion-exchange chromatography by **Chen *et al*,.(2004)**.

Table 12: Purification and Protein determination

S.No.	Purification Step	Total Activity	Total Protein Content(mg)	Specific Activity (U/ml)	Purification fold	Yield (%)
1	Crude	4537.2	1633.17	2.77	1	100
2	Ammonium Sulfate	1411.62	489.65	2.88	1.04	31.11
3	Dialysis	787.65	214.56	3.67	1.32	17.35
4	Ion-exchange	323.52	77.26	4.18	1.50	7.13

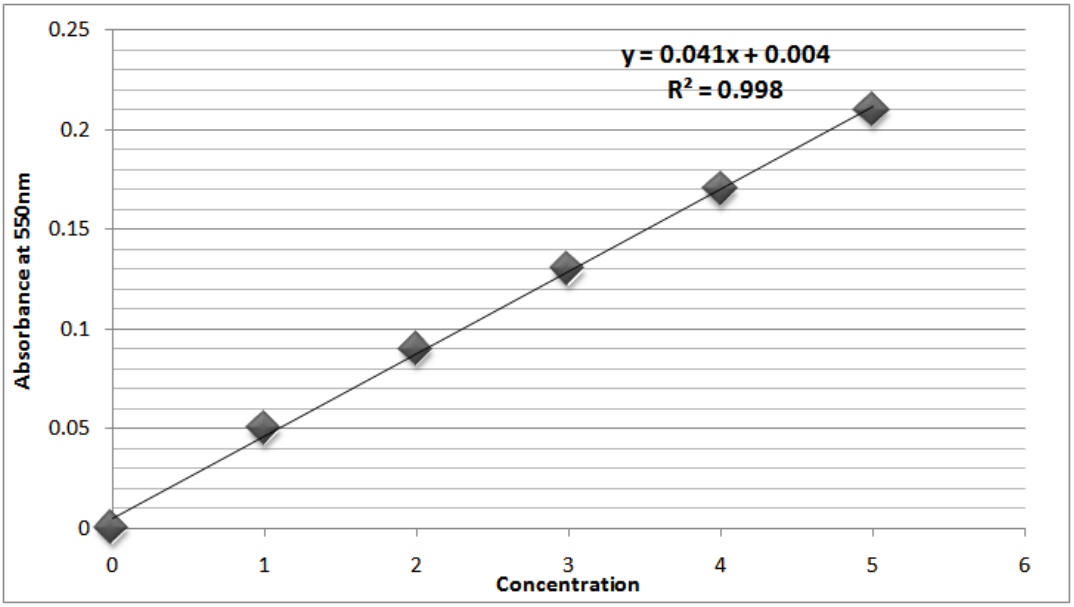

Fig.25 Standard Curves for protein determination

4.7 CHARACTERIZATION OF PURIDFIED CELLULASE

Effect of pH on Cellulase Activity

Effect of different pH values ranging from 4-9 was studied on cellulase enzyme. The purified enzyme was mixed with 0.01M phosphate buffer at different pH. Enzyme assay was performed after 15min of incubation using carboxymethyl cellulase as substrate on spectrophotometer at wavelength of 550nm revealed that the cellulase enzyme was completely active at large pH (6-7.0) range. and presented an optimum activity of 5.86U/ml at a pH value of 7.0, Further increase in pH from optimum value (pH 7.0) showed decreasing trends in its activity.

Findings of the present study are in accordance with**Irfan *et al*.,(2012**) who observed optimum pH ofcellulase activity at pH 7.5 (0.451± 0.011 IU/ml/min). **Yin *et al,*. (2010)** isolated *Cellulomonas* sp. YJ5 showing its optimum pH of 7.Cellulase enzyme of *B. subtilis subsp. subtilis* A-54 has optimum pH of 6.5 **Kim *et al*.,(2009).**According to previousstudies, cellulases are active at the pH range of 6.0to 7.0 from *A. Niger* **Akiba *et al*.,(1995)**5.0 to 7.0 from *Lysobacter spp.***Ogura *et al*.,(2006)** and 5.0 to 6.5 from *Bacillus* strains **Mawadza *et al*.,(2000)**. The data were analyzed statistically using t-statistics, line regression and the result showed non-significant result at 5% probability level. (App IV-3.5)

Table13: Effect of pH on cellulase activity

S.No.	1	2	3	4	5	6	7	8	9	10	11
Incubation pH	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
Enzyme Activity(U/ml)	2.46	3.08	3.69	4.00	4.62	4.79	5.86	4.31	3.39	2.77	1.54

r =0.12, $t_{(cal)}$ = 0.36 < $t_{(tab)}$ = 2.22 at 5% level (Non Significant) y = 1.763x + 0.332

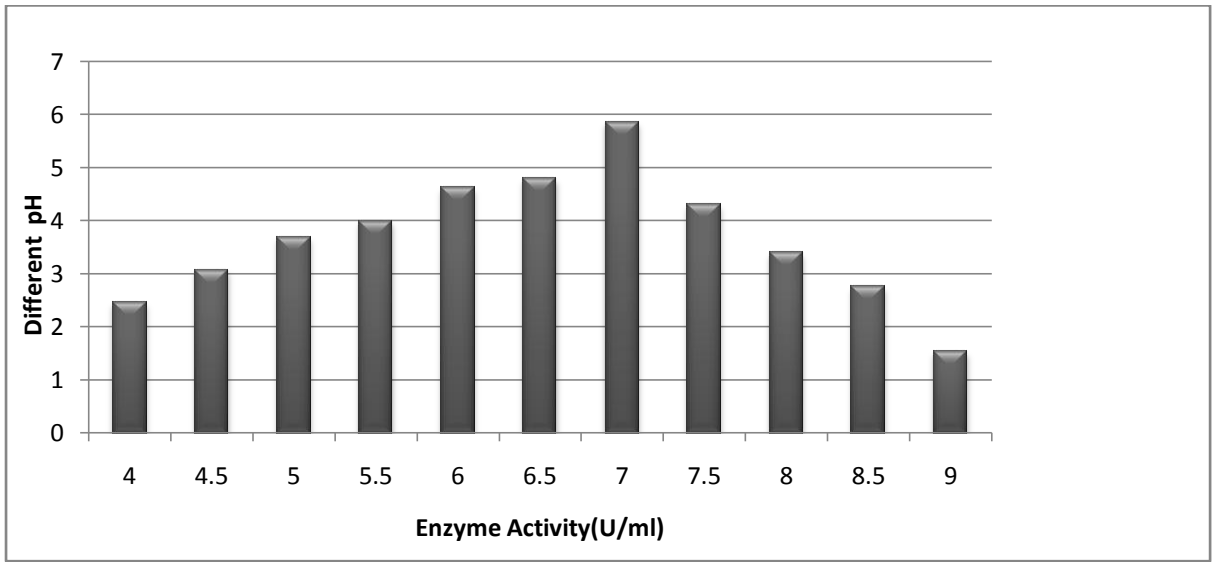

Fig.25:Characterization of Cellulase at different pH

Effect of temperature on cellulase activity

Effect of different temperature (25-60ºC) on the purified enzyme was determined by incubating at different temperature under controlled condition. Cellulase was used to determine effect of temperature on enzyme activity by incubating it for 15 min. Maximum activity was observed at 50ºC (fig.4.9) above which it rapidly lost activity and became denatured as the denaturation of the enzymic protein occurs at elevated temperatures. Findings of the present study are in close agreement with the findings of Thongekkaew et al. (2008) who reported 40 - 50°C as an optimum temperature during the characterization of cellulase produced from Cryptococcus sp. S-2. The temperature optima for total cellulase activity was found at 50°C Ozioko et al.,(2013). Whereas, Fadel (2000) found 55°C as a best temperatureat which the enzyme was most active and stable. Saha (2004) also reported the sametemperature i.e. 55°C as optimum for cellulase activity. The optimum temperature of purified cellulase was lower than some of other Bacillus strains [65°C (CH43) and 70°C (RH68)Mawadza et al., (2000).The data were analyzed statistically using t-statistics, line regression and the result showed non-significant result at 5% probability level. (App IV-3.4).

Table. 14: Effect of temperature on cellulase activity

S.No.	1	2	3	4	5	6	7	8
Incubation Temperature	25°C	30°C	35°C	40°C	45°C	50°C	55°C	60°C
Enzyme Activity(U/ml)	4.46	7.28	12.39	22.83	27.77	40.12	19.87	16.32

r =0.58, $t_{(cal)} = 2.23 < t_{(tab)} = 2.36$ at 5% level (Non Significant) y = 1.763x + 0.332

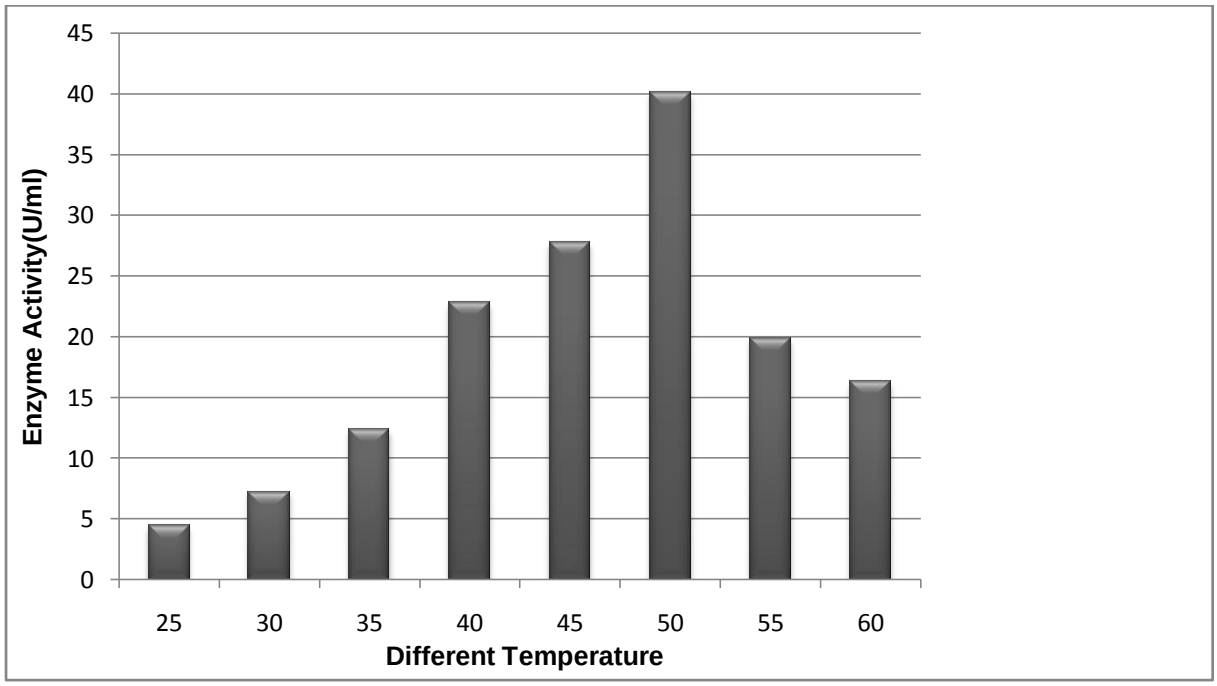

Fig.26:. Characterization of Cellulase at different incubation temperature

INDUSTRIAL APPLICATION

Detergent compatibility of purified cellulase

Detergent compatibility of purified cellulase was determined against different detergent.

Enzyme incubated at 50°C with detergent solution for 15 minutes revealed maximum Compatibility with tide detergent followed by Nirma (figure) revealing that the cellulase is Compatible with local detergents. Similarly **Iqbal *et al*.,(2011)** tested four different detergents brands (surf excel, Ariel, wheel,Bright) for checking the compatibility of cellulase and found maximum compatibility with Surf Excel. The data analyzed statistically using f-statistics and the result showed significant result at 5% probability level.

Table. 15: Detergent compatibility with different detergent brands.

S.No	1	2	3	4
Detergent Brands	Tide	Nirma	Ghari	Fena
Enzyme Activity(U/ml)	81.78	70.36	49.9	24.75

Due to detergent: $F_{(cal)} = 8.50 > F_{(tab)} = 3.18$ (Significant) at 5% level

Due to enzyme: $F_{(cal)} = 12.04 > F_{(tab)} = 3.18$ (Significant) at 5% level

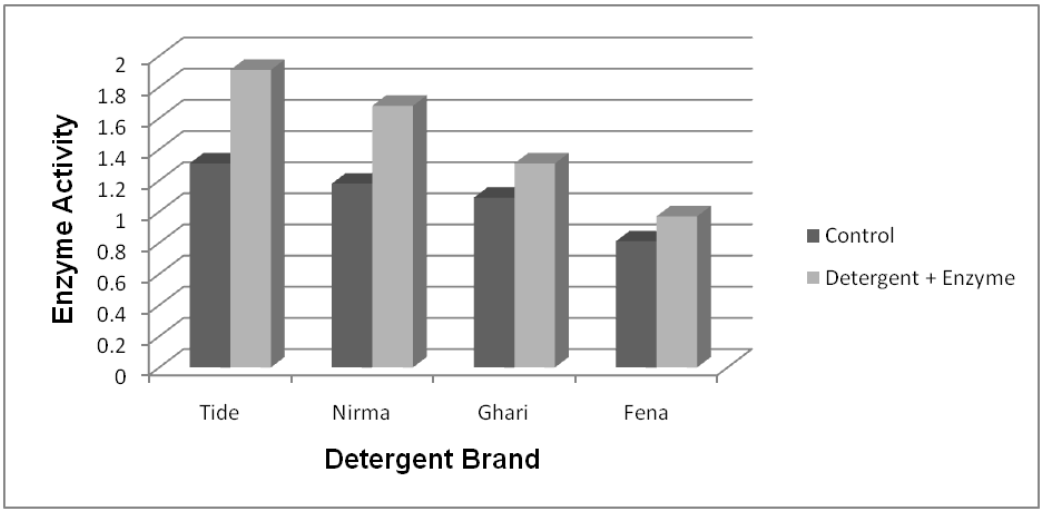

Fig.26: Detergent compatibility with different detergent brands.

IV. SUMMARY AND CONCLUSION

In this study, *Bacillus cereus, Bacillus Pantothenticum, Bacillus Insolitus, Bacillus Insolitus, Bacillus alvei, Bacillus Firmus, Bacillus alvei,Bacillus alcolphilus, Bacillus alvei,* and *Bacillus subtillus* was isolated from the different region of Allahabad. Garden soil, wood furnishing region, sugarcane farm soil, chimney region and petrol region. All the isolated bacterial culture was identified on the basis of cultural, morphological and biochemical characteristics. The substrate used for cellulase production were sugarcane bagasse, rice straw, Chana husk, wheat straw and Ground nut cell. Among them the best substrate for maximum production was done on sugarcane bagasse with *Bacillus cereus*and optimizations were performed on the same. The enzyme was extracted and assayed with different optimized parameter *i.e.* Incubation period, initial pH, temperature, different carbon source and nitrogen sources. Purification was done by ammonium sulphate precipitation, dialysis and ion exchange chromatography. After that protein determination of both crude and purified enzyme was done by Lowry method. Characterization was done with different parameter *i.e.* initial pH and temperature. Finally industrial application was done to check the compatibility of cellulase with different detergent brands. From the study following observations were made and conclusions were drawn:

- An analysis of 100 soil sample 40(40%) cellulytic bacteria were obtained..
- On the basis of morphological, cultural, biochemical and molecule characteristic the different bacillus species are identified as
- For maximum cellulose production the effective cellulolytic strains of Bacillus cereus required an optimum temperature (30ºC) on 4^{th} day (0.38U/ml).
- The major peak of activity was found after 72 hrs. Incubation beyond the optimum time showed a rapid decline in the enzyme yield, as compare to maximum (0.173U/ml) at 120 hrs.
- Beyond the optimum temperature 30°C, a sharp fall in cellulase activity was obtained. The cellulase activity was minimum (enzyme activity 5.55U/ml) at 25°C and maximum (enzyme activity 43.09U/ml) at 30°C.
- In present study the optimum pH 7.5 (enzyme activity14.69U/ml) was reported for cellulase production from Bacillus cereus. The minimum cellulase production was at pH 4.0 (enzyme activity 0.38U/ml). Whereas the enzyme activity declines on either side of the optimum pH value.
- Among all the Nitrogen sources peptone produced maximum cellulase activity (enzyme activity 19.95U/ml) and minimum Glycine (enzyme activity 1.2U/ml).
- Among all the Carbon Sources Lactose produced maximum (enzyme activity 30.63U/ml) and minimum Glucose (enzyme activity 14.7U/ml).
- The total activity of crude enzyme and total activity of chromatography enzyme showed a 4537.2 and 323.52 respectively. The total protein content of crude and total protein content of Ion-exchange chromatography showed a 1633.17mg/ml and 77.26mg/ml. DEAE-cellulose chromatography resulted in purification fold is 1.50.
- The effect of pH on cellulase activity was characterized, enzymatic activity were found for maximum at pH 7.0 (5.86U/ml) and minimum enzymatic activity at pH 9.0(1.54U/ml).
- The effect of temperature on cellulase activity was characterized, maximum enzymatic activity was found for at 50°C (40.12U/ml) and minimum enzyme activity found at 25°C (4.46U/ml).
- The four local detergent brands were used to checked the compatibility of cellulase enzyme in which maximum compatibility were examined for Tide detergent (enzyme activity 81.78U/ml) and minimum for Fena detergent (enzyme activity 24.75U/ml).

The present study was satisfactory focusing on the affinity of the enzyme with different substrate in increasing process efficiency. In this study bacillus cereus found to be the best cellulase producing bacteria and it can be implemented at industrial level (food, feed, textile, detergent industries and pulp and paper industries etc.). Cellulose-hydrolyzing enzyme has found various biotechnological applications. Cellulolytic enzyme can be used in alcohol production to improve juice yield and effective color extractions of juices. The presence of cellulase in detergents cause color brightening softens and improves particulate soil removal. Cellulose degrading bacteria has high potential to produce electrical energy which can be utilized in microbial fuel cell (MFC). Cellulase is used extensively in the isolation of plant protoplasts, frequently in combination with maccrozyme R10. To utilize the agro-biomass waste products Bacillus Cereus can be used as an efficient agent.

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