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Production, optimization and purification of bacterial cellulase by solid state bioprocessing of agro biomass

Ajay Kumar Singh [1], Abhas Kumar Maharana[1], Harison Masih[1], Yashab Kumar[1], Santosh Kumar Mishra*[2]

[1]Department of Microbiology & Fermentation Technology, Sam Higginbottom Institute of Agriculture, Technology & Sciences(Deemed to be university),Allahabad U.P. INDIA
[2] Department of Biotechnology, IMS Engineering College, Ghaziabad U.P. INDIA

ABSTRACT

A total of 25 soil samples were collected from different sites of Allahabad. On the basis of maximum hydrolytic zone formation on CMC agar media it has been observed that 10 isolates were found positive for cellulase production.. The isolates that showed maximum cellulase production was identified as Bacillus circulans. Cellulase was produced by Bacillus circulans with banana peel as solid substrate. The different process parameters for cellulase production were evaluated . The optimum condition for cellulase production were 25°C and 7.0 pH. Optimum moisture was 65% and inoculum size was 0.5ml (6×10^6 cfu/ml) for maximum production of cellulase. Crude enzyme was partially purified by ammonium sulphate precipitation, dialysis, and DEAE-cellulose ion exchange chromatography. The crude and purified enzyme showed enzyme activity of 0.56 U/ml and 0.58 U/ml respectively. The purified enzyme had 3.167 mg/ml protein where as the crude had 9.75 mg/ml. DEAE-Cellulose chromatography resulted in purification fold of 3.18.

Keywords: Bacillus circulans, cellulase, DEAE-Cellulose chromatography

Corresponding author
Email: skmiet@gmail.com

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INTRODUCTION

Cellulase is a hydrolytic enzyme which breaks down cellulose into smaller oligosaccharides and glucose. Cellulose constitutes the largest supply of biomass material and 20-45% of cellulose is present in plant tissue in dry weight. Cellulases are among the industrially important hydrolytic enzymes and are synthesized by microorganisms during their growth on cellulosic materials. Microbial conversion of cellulosic / lignocellulosic biomass into useful products is a complex process involving combined action of three enzymes namely endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) [12].

Cellulases are widely used in the food, feed, textile, pulp and paper industries [27]. Cellulose-hydrolyzing enzymes are widespread in fungi and bacteria. The most effective enzyme of commercial interest is the cellulase produced by Trichoderma spp. [35]. A preliminary study showed that Bacillus subtilis (CBTK 106) can produce a considerable amount of cellulase activity [19].

Bacteria belonging to Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora, and Streptomyces can produce cellulases [7]. Cellulomonas fimi and Thermomonospora fusca have been extensively studied for cellulase production. Although many cellulolytic bacteria, particularly the cellulolytic anaerobes such as Clostridium thermocellum and Bacteroides cellulosolvens produce cellulases with high specific activity, they do not produce high enzyme titers [10]. Because the anaerobes have a very low growth rate and require anaerobic growth conditions, most research for commercial cellulase production has focused on fungi [10]. There are few bacteria such as Cellulomonas, Clostridium, Sinorhizobium fredii, Bacillus spharicus, Bacillus circulans, Pseudomonas, Paenibacillus azotofixans, Gluconacetobacter, Azospirillum, Cytophaga, Vibrio and Ruminococcus etc. which are cellulolytic [11]. Extra cellular cellulase enzymes, often involved in breaking up of polymers and complex organic molecules, are themselves subjected to sorption and deactivation by soil colloids [34]. These enzymes can either be free, particularly in aerobic microorganisms or grouped in a multi component enzyme complex, cellulosome, such as in aerobic cellulolytic bacteria [6].

Production of cellulase in SSF using various substrates, microorganisms, and nutrient solutions has been reported [22, 25, 33]. Solid state fermentation (SSF) offers advantages over fermentation in liquid broth (submerged fermentation) like higher product yield, better product quality, cheaper product recovery and cheaper technology [28]. The direct applicability of the product, the high product concentration, and the reduced costs of dewatering make SSF a promising technology for cellulase production [36]. Much of the cellulose in nature exists as waste material from agriculture industry in the form of husk, stalks, stems and peels. So to utilize these waste products and to develop cheaper method for production of cellulase enzyme for enzymatic degradation the present study describes the production, optimization and purification of bacterial cellulase by solid state bioprocessing of agro biomass.

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MATERIAL AND METHODS

Isolation and screening of cellulolytic bacteria

Soil samples were collected from different places of Allahabad(U.P), India. These soil samples were serially diluted up to 10^{-6} and spreaded over the Caboxymethyl Cellulose (CMC) agar medium plates and incubated for 72 hrs. at 25 ±1°C [26]. 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 15 min. 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

The selected strain was grown on nutrient agar slants at 25 ±1°C for 72 hrs. and maintained on nutrient agar slants at 4°C. For the identification of strain of interest cultural characteristics, morphological characteristics, and biochemical tests were conducted and identified on the basis of characters as given in Bergey's Manual of Systematic Bacteriology [15].

Substrates and its pretreatment

In the present study, the different substrates such as banana stem, banana peel, banana leaf, and wheat straw were used. Substrates were sliced, spreaded on trays and oven dried at 70 ±2°C for 24 hrs. The dried slices were grounded and sieved through standard mesh sieves to obtain particles ranging in size from 200- 2400 µm, and stored in polyethylene bags at room temperature (30±2°C) until use.

Production of cellulase

Production medium and conditions for control

The bacterial strain was grown in 250 ml Erlenmeyer flasks containing each of various substrates (wheat straw, banana peels, banana leaf, and banana stem) and moistened with mineral salt solution [gl^{-1}: Na_2 HPO_4. $2H_2O$, 1.1; NaH_2 PO_4 .$2H_2O$, 0.61; KCl, 0.3; $MgSO_4.7H_2O$, 0.01; pH 7.0]. It had an initial moisture content of 75% and was autoclaved at 121°C for 60 min, cooled to about 30°C and inoculated with an inoculum of 3ml. The contents of the flasks were mixed thoroughly to ensure uniform distribution of the inoculum and were incubated at 35°C for 120hrs. The enzyme was then extracted and assayed [20].

Enzyme extraction

The enzyme from fermented substrate medium was extracted with 0.01M phosphate buffer (pH 7.0) applying a substrate: buffer ratio of 1:10 employing a simple contact method [19]. The enzyme extract was centrifuged at 8000 g for 20 min. at 4°C. The clear supernatant obtained was used for the assay.

Crude enzyme assay

The enzymatic activity of crude enzyme was determined by estimating the reducing sugar produced during enzymatic reaction by 3, 5-dinitrosalicylic acid method [24]. One unit of activity is the amount of enzyme required to release 1.0 µmol of reducing sugar (as glucose) per minute under the described conditions.

Optimization of production parameters

The various process parameters that influence the enzyme production during SSF were optimized over a wide range. Process parameters such as incubation period, initial pH , incubation temperature, different carbon sources, different nitrogen sources, moisture content, and inoculum size were optimized for maximum enzyme production in triplicates [20].

Purification of crude enzyme

After 96hrs. production, the enzyme was extracted and extract was purified by the following techniques.

Ammonium sulphate precipitation

Ammonium sulphate was added to cell-free supernatant to give 80% saturation at 4ºC. The mixture kept overnight and the resulting precipitate was collected at 8000 rpm for 15 min. [4].

Dialysis

The precipitate collected was dissolved in minimum volume of 0.01M phosphate buffer (pH 7.0) and dialyzed against same buffer for 24 hrs. at 4ºC [4].

Ion-exchange chromatography

The solution after dialysis was applied on a DEAE-cellulose column equilibrated with 0.01M phosphate buffer at pH 7.0. The column was packed with the DEAE-Cellulose solution and then the column was so equilibrated so as the rate of flow was equal to 1 $ml.min^{-1}$. Then the sample was loaded and the buffer was added and step wise elution was done with sodium phosphate buffer, pH 7.0, from 0.1-1.0 M sodium chloride gradient. All the samples eluted were

collected and the cellulase activity was checked and determined for each fraction by cellulase assay procedure [4].

Protein estimation

Protein of both crude and purified enzyme was estimated by using bovine serum albumin (BSA) as standard [21].

RESULTS AND DISCUSSIONS

Isolation

A total of 25 soil samples were collected from different places of Allahabad(U.P) India, such as from saw mill, agriculture field, petrol pump, river bank and chimney. Incidences of cellulolytic bacteria were detected from all the places except chimney. Maximum incidences (50%) of cellulolytic bacteria were found to be present in agriculture field soil samples. The incidence of cellulolytic bacteria from different sites of Allahabad was different due to many reasons like soil environment, season, and time of collection of soil samples. The incidence of cellulolytic bacteria is in agreement with the present study [1, 11, 23].

The selected strain was identified as Bacillus circulans (isolated from agriculture field) by its cultural, morphological and biochemical characteristics. Bacillus circulans was found creamy, circular, entire, flat, opaque and small rod chains, gram positive in cultural and morphological characteristics. Bacillus circulans showed all positive results in sugar fermentation test. In the starch hydrolysis, and catalase activity B. circulans showed positive results and negative in all other biochemical tests.

Selection of best substrate

In the present study banana stem, banana peel, banana leaf, and wheat straw were used as substrate. Among all the substrates banana peel gave the maximum cellulase production (0.206 U/ml) on 4^{th} day when fermented with Bacillus circulans under SSF (Fig. 1). Wheat straw was found as a poor substrate among others.

The total enzyme titres were 4-6 folds higher in banana peel than those when banana stem, banana leaf and wheat straw were used as substrates. Total cellulase production by banana stem and banana leaf was more or less the same. These results support the suitability of using banana wastes as solid substrate for high production of cellulases [20].

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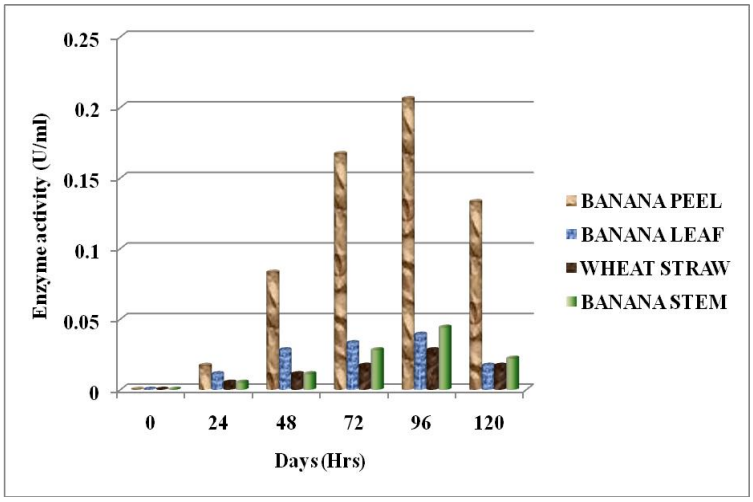

Fig. 1: Selection of best substrates for production of cellulase enzyme

Optimization of media parameters for maximum cellulase production from Bacillus circulans

Effect of incubation period

During the course of study, the activity of Bacillus circulans was detected in the production medium supernatant from the first day to fifth day. The major peak of activity was found after 72hrs. Incubation beyond the optimum time showed a rapid decline in the enzyme yield, as compared to maximum (0.206 U/ml) at 96hrs. (Fig. 2).

The reduction in cellulase yield after an optimum period is probably due to depletion of nutrients available to micro-organisms. A maximum of 96 hrs. was reported for the optimal cellulase production by Bacillus circulans [30]. Bacillus spp. B21, Bacillus pumilus and Bacillus subtilis showed maximum cellulase activity at 72hrs. of incubation period [3, 14].. However, this was in contrast with the finding of many other workers, who reported maximum cellulase productivity after 36 hrs. by Arachnoitus spp. [2], 72 hrs. by Bacillus subtilis by using banana stalk as solid substrate [32], 120 hrs. by Bacillus pumilus [29], and 142 hrs. incubation by Clostridium cellulolyticum [13].

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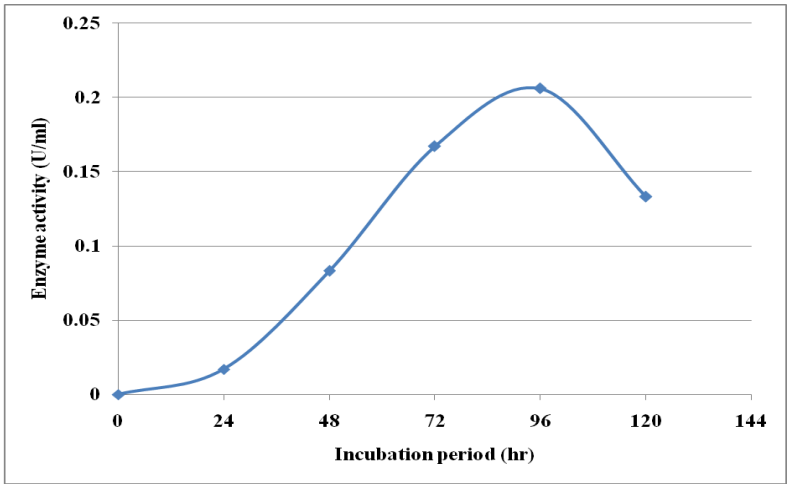

Fig. 2: Production of cellulase from Bacillus circulans at different incubation period

Effect of incubation temperature

The data obtained during the course of study indicated that there was a significant effect of temperature on cellulase production. Beyond the optimum temperature 25°C, a sharp fall in cellulase activity was obtained. The cellulase activity was minimum (0.006 U/ml) at 55°C and maximum (0.261 U/ml) at 25°C (Fig. 3).

The optimum temperature of 25°C was found to maximally influence the microorganism Bacillus circulans to produce the cellulase [26]. The mutant Bacillus pumilus BpCRI 6 shows maximum cellulase activity at 25°C [18]. However, these results were in contrast with the data recorded by many other workers. Optimum temperature recorded for maximum cellulase productivity was at 30°C for Cellulomonas spp. and Bacillus pumilus [29], 35°C for Bacillus subtilis CBTK 106 and Bacillus spp. B21 [3,20,32] and 37°C for Bacillus spp., Clostridium cellulolyticum Ce19M and Pseudomonas flourescens [5,14].

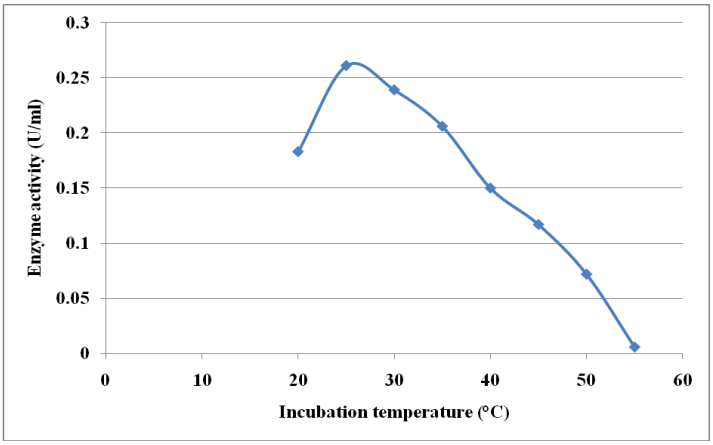

Fig. 3: Production of cellulase from Bacillus circulans at different incubation Temperature

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Effect of initial pH

The data obtained indicates 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 7.0 (0.217 U/ml) was reported for cellulase production from Bacillus circulans. The minimum cellulase production was at pH 10 (0.028 U/ml) (Fig. 4).

Maximum cellulase productivity was found at pH 7.5 using Bacillus circulans [30]. Many reports were found showing the maximum productivity at 7.0 pH using Bacillus subtilis [32], Cellulomonas, Bacillus, and Micrococcus spp. [16]. Bacillus licheniformis cellulase was found to be more stable under acidic conditions [8]. Alkalophillic Bacillus spp. cellulases have been reported to have optimal activities between pH 8 and 9 [31].

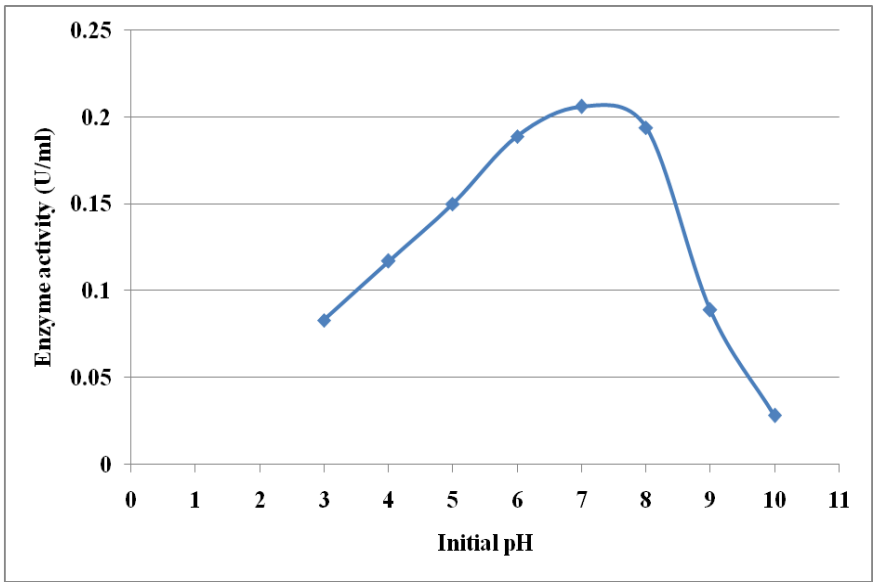

Fig. 4: Production of cellulase from Bacillus circulans at different initial pH

Effect of moisture content

The data obtained during the course of time of study indicated that there is a significant effect of moisture content on cellulase production. The cellulase activity was found maximum (0.272 U/ml) at 65% of moisture content and minimum (0.094 U/ml) at 85% (Fig. 5).

The appropriate moisture of substrate is one of the critical factors influencing the solid-state fermentation (SSF), and is governed by the requirements of the micro-organism. However, these results were in contrast with the data recorded by many other workers. Optimum moisture content recorded for maximum cellulase productivity was 70% for Bacillus subtilis [32, 38].

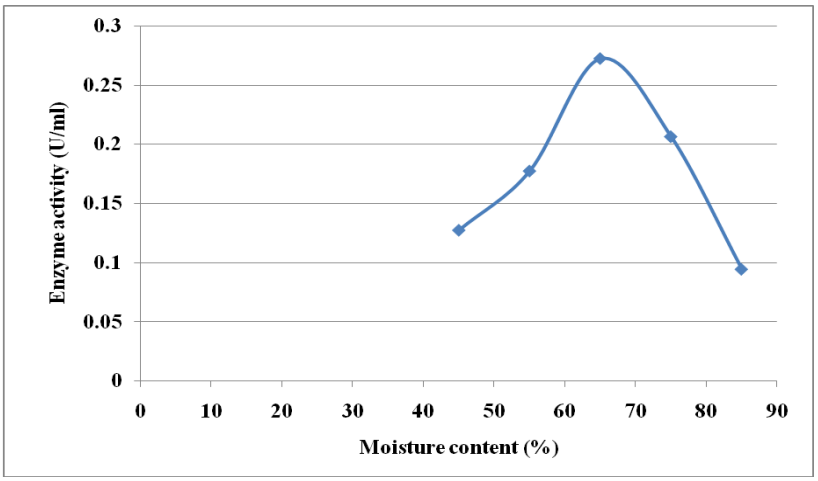

Fig. 5: Production of cellulase from Bacillus circulans at different moisture content

Effect of inoculum size

The data obtained indicates there is strong influence of inoculum size on cellulase production. The cellulase activity was decreased as the inoculum size increased. The maximum (0.322 U/ml) cellulase activity was at 0.5 ml inoculum size (6× 10^6 Cfu/ml) and minimum (0.106 U/ml) at 3.0 ml. inoculum (36× 10^6 Cfu/ml) (Fig. 6).

They found that small inoculum size controls and shortens the initial lag phase whereas larger inoculum size increased the moisture content to significant extent. The free excess liquid presents an additional diffusion barrier together with that imposed by solid nature of the substrate and leads to a decreased in growth and enzyme production [17, 25, 37]. However, this was in contrast with the finding of many other workers, whom recorded maximum cellulase productivity in 5ml of Bacillus subtilis[32].

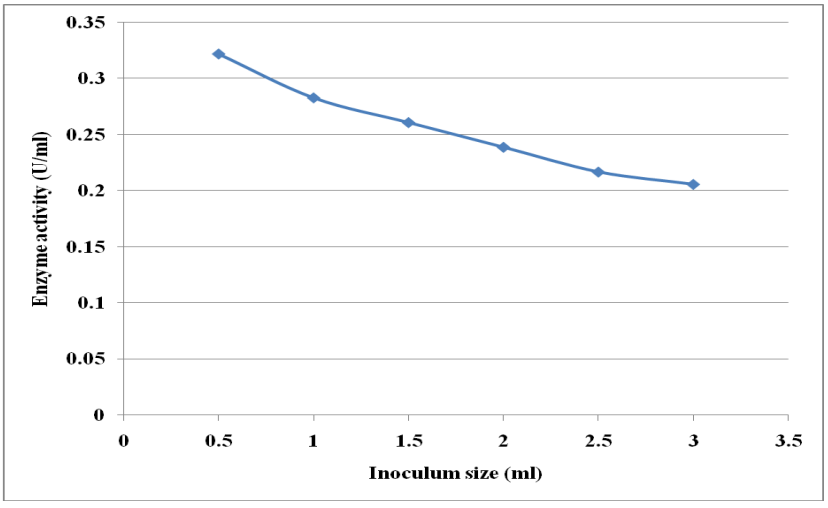

Fig. 6: Production of cellulase from Bacillus circulans at different inoculum size

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Effect of nitrogen sources

The enzyme activity was studied for different nitrogen sources at different concentration of 1%, 2%, and 3%. Among these nitrogen sources $(NH_4)_2SO_4$ produced maximum cellulase activity as compared to $NaNO_3$. The maximum activity was showed at 3% of $(NH_4)_2SO_4$ (0.361 U/ml) and minimum activity was at 1% of $(NH_4)_2SO_4$ (0.206 U/ml) (Fig. 7). Production of cellulase was enhanced by the additional nitrogen sources like $(NH_4)_2SO_4$, peptone and yeast extract in Bacillus subtilis and Bacillus circulans [30].

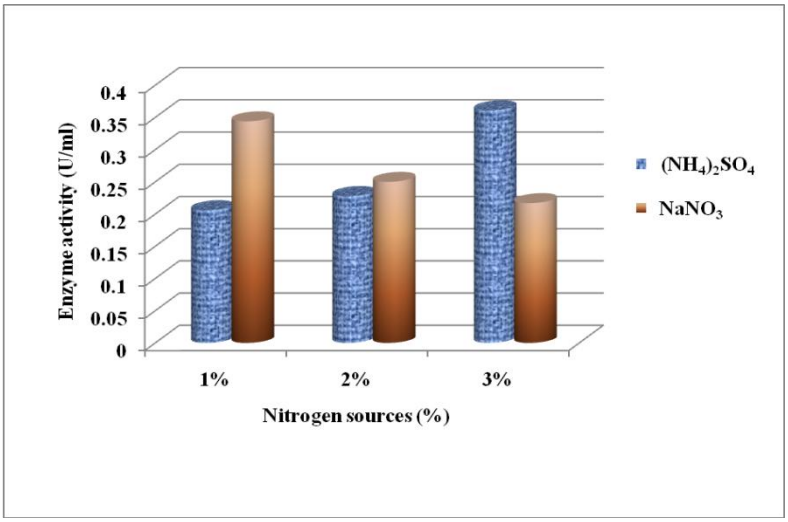

Fig. 7: Production of cellulase from Bacillus circulans at different nitrogen sources

Effect of carbon sources

The enzyme activity was studied for different carbon sources at different concentration of 1%, 2%, and 3%. Among these sugar sources sucrose produced maximum cellulase activity as compared to lactose. The maximum activity was showed at 3% of sucrose (0.517 U/ml) and minimum at 1% of sucrose (0.183 U/ml) (Fig. 8).

For maximum production of cellulase, Bacillus circulans utilize higher concentration of sucrose in the production medium. Production of cellulase was enhanced by the additional carbon sources like cellulose, sucrose, cellobiose etc. in Bacillus subtilis and Bacillus circulans [30].

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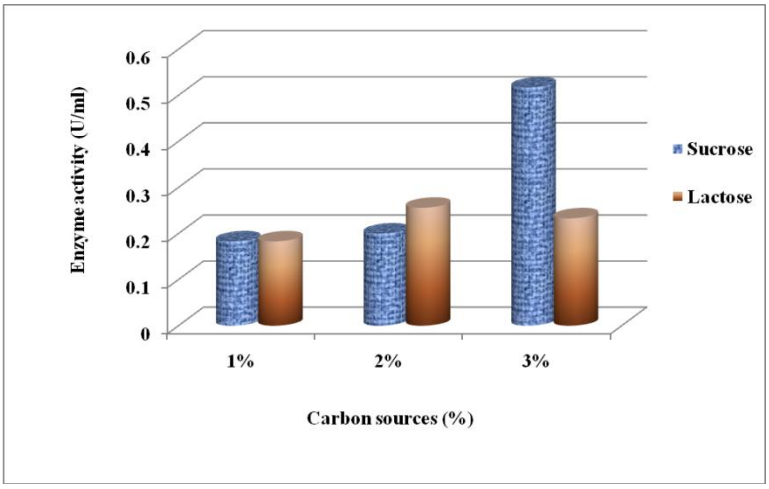

Fig. 8: Production of cellulase from Bacillus circulans at different Carbon sources

Cellulase purification and protein determination

From the maximum yielding condition the cellulase was produced by Bacillus circulans. The crude enzyme showed the enzyme activity of 0.56 U/ml. The crude enzyme was purified by precipitating it with different concentrations of ammonium sulphate (40, 50, 60, 70 and 80). It was observed that 80% concentration of ammonium sulphate showed better performance for the enzyme precipitation. The recovery was further followed by dialysis and ion exchange column chromatography yielding the highest enzyme activity [4]. The elution was done with gradient ranging from 0.1-1.0M NaCl, and the highest activity was yielded with 0.4M NaCl concentration (0.58 U/ml). However, Pseudomonas fluorescens cellulase was purified at 90% ammonium sulphate saturation [5].

Protein of both crude and purified enzyme was estimated by using bovine serum albumin as standard [21]. The yield was gradually decreases in every purification steps. The purified enzyme had 3.167 mg/ml protein where as the crude had 9.75 mg/ml. DEAE-Cellulose chromatography resulted in purification fold of 3.18 (Table 1). Bacillus strain M-9 that DEAE-Cellulose chromatography resulted in purification fold of 3.47 to 9.06 [4]. Sinorhizobium fredii cellulase was purified by 9.08 folds using ion exchange chromatography [9]. Similarly, Pseudomonas fluorescens cellulase was purified by 24 to 25 folds by using ammonium sulphate precipitation and ion exchange chromatography [5].

Table 1: Purification and protein determination

Steps	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific activity (U/mg)	Purification fold
Crude Enzyme	200	112.22	1950	0.058	1
Purified Enzyme	10	5.83	31.67	0.184	3.18

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CONCLUSION

In this study Bacillus circulans found to be the best cellulase producing bacteria and it can be further optimized at large scale level (food, feed, textile, pulp and paper industries etc.). The results indicated the suitability of cheaper and abundantly available banana peels waste as solid substrate for large-scale production of cellulase in an SSF system, thereby minimizing the high costs when other substrates, and chemicals, are used for cellulase production. Total enzyme titres with banana peel as solid substrate were 3-6 folds higher than among all other substrates. Maximum utilization of this waste can also contribute to efficient solid-waste management, where continuous accumulation of agricultural wastes poses a serious environmental problems.

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