OPTIMIZATION OF CARBOXYMETHYLCELLULASE PRODUCTION VIA SUBMERGED FERMENTATION FROM A NEWELY ISOLATED STRAIN OF *Bacillus subtilis* subsp. *inaquosorum*

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Abstract— **Fossil fuels reserves are depleting at an alarming rate which has developed a grim situation globally drawing the attention of scientific world. The rising costs of fuels have shifted the concerns to exploit renewable sources for production of value added chemicals. Worldwide research is going on to utilize the natural cellulosic biomass efficiently as it is considered as a virtually inexhaustible source of raw material to develop novel processes and products. But main obstacle in the path of success is high cost of enzymes necessitating the need to develop economically profitable processes. Cellulases are drawing the attention of the scientific world by virtue of their immense potential in lignocellulosic biomass utilization. The need for isolation of novel microorganisms from diverse habitats is imperative as they provide a platform for genetic modifications. In the present study, a novel strain of cellulose degrading bacteria was isolated from soil and identified as *B. subtilis* subsp. *inaquosorum*. Optimization studies were performed to enhance carboxymethylcellulase production. Maximum CMCase (1.4 IU/ml) activity was observed at 30ºC, 150 rpm after 72 hrs using wheat bran as carbon source.**

***Keywords*— *Bacillus subtilis* subsp. *inaquosorum*, CMCase, Optimization, Submerged fermentation, Wheat bran**

I. INTRODUCTION

Lignocellulose represents a major component of woody as well as non-woody plants. It is a substrate which holds enormous biotechnological potential to be exploited as renewable source for production of value added chemicals. Due to various agricultural and industrial processes, a large amount of lignocellulosic waste is generated posing a threat to environment. The judicial utilization of these wastes to produce industrially relevant compounds will not only solve the problem of environmental pollution but also provide an economically efficient substrate for industry based exploitation. Structurally, it consists of three components: lignin, hemicellulose and cellulose.

Cellulose is an eminent representative of naturally available nanostructure matter. Cellulose is synthesized primarily by plants; however some microorganisms, animals and algal species can also produce it [1], [2]. Cellulose has a complex multilevel architecture made up of bundles of microfibrils. Each microfibril may contain 36-1200 cellulose chains which are held together by hydrogen bonds and van der Waal forces ultimately resulting in a highly ordered crystalline structure. Each cellulose chain is a non-branched chain of D-glucose monomers that may range from 100 to 20000 linked together by β-1, 4 glycosidic bonds [3]. The repeating unit of cellulose is cellobiose, a disaccharide not glucose in contrast to other glucan polymers. These well organized crystalline structures are interspersed with disordered or disorganized domains (amorphous cellulose) constituting 5-20 % of the micro fibril. Cellulose exists in the form of sheets of glucopyranose rings lying in a plane with sheets stacked on one another to form a 3-D particle. The surface of the particle has several "faces" due to this arrangement that interact with the aqueous environment and the cellulolytic enzymes [3], [4]. Hydrogen bonding interactions in these amorphous regions are suboptimal, thus accessible for water molecules and enzymatic attack [5].

Biomass-derived energy products including ethanol, hydrogen, methanol etc. are considered as important members of biofuels. The demand for ethanol has been accelerating dramatically enhancing the market value owing to its potential utilization as a chemical feedstock or petrol additive or as an octane enhancer [6]. In U.S.A., ethanol is produced from corn and constitutes about 99% of the biofuels [7]. Various crop residues like rice straw, wheat straw, sunflower stalks, corn cob, sunflower hulls and water hyacinth have been utilized efficiently for bioethanol production [8]-[10]. Microbial conversion of biomass serves an efficient method to produce value added chemicals. Cellulase market is accelerating dramatically worldwide. Cellulases are produced by a variety of microorganisms including actinomycetes, bacteria and fungi. A lot of reviews have listed the different types of microorganisms involved in degradation of cellulose [11]-[14].

Screening and isolation of microorganisms with cellulose degrading activity is of quite importance so as to get novel

cellulases. The newly isolated microbes with some unique characteristics can act as platform for further improvement. Generally cellulase producing organisms are isolated from soil samples obtained from forests, agricultural fields, hot water springs, animal manure and other natural reserves. Keeping this in mind, cellulose degrading bacteria has been isolated from soil and optimization studies were carried out to enhance cellulase production.

II. MATERIAL AND METHODS

A. Isolation of Cellulose Hydrolysing Bacteria

The soil samples collected from different locations were diluted serially with sterile water. Different bacterial strains were isolated by serial dilution method and pour plate method. Each dilution (100 µl) was spread on agar plates followed by incubation at 37^0C for 24 hrs. Isolates were purified by repeated streaking and preserved for further experiments.

B. Screening for Cellulose Hydrolysing Bacteria

The isolates were spot inoculated on agar plates supplemented with 1% (w/v) carboxymethylcellulose (CMC) and incubated further for 48 hrs at 37^0C. Plates were covered with aqueous solution of 1 % Congo red dye at room temperature for 20 min and then counterstaining of the plates was performed by thorough washing with 1N NaCl. Cellulase activity was indicated qualitatively by the appearance of clear hydrolysis zone around colonies possessing cellulose hydrolyzing ability [15]. For each isolate, Hydrolysis capacity (HC) ratio was calculated by dividing the zone diameter by colony diameter. Isolates with higher HC ratio were selected for quantitative determination of CMCase activity and identification. Gram staining and morphological examination of all the cellulose degrading isolates was also performed.

C. Enzyme Production by Submerged Fermentation

A single colony of the selected isolates was inoculating a into 100 ml Erlenmeyer flask containing 20 ml nutrient broth media and primary culture was prepared by incubating at 37°C, 150 rpm for 24 hrs. 100 ml of nutrient broth media supplemented with 1% wheat bran as carbon source in a 250 ml Erlenmeyer flask was prepared and sterilized. The production medium was inoculated with 1% (v/v) primary culture and incubated at 37°C for 48 hours with 150 rpm agitation speed in an orbital Incubator-shaker. The centrifugation of the culture was carried out at 4°C, 8000×g for 20 minutes to pellet down bacterial cells as well as other unwanted materials. The cell free supernatant obtained after centrifugation served as a source of crude enzyme and was preserved at -20°C until further analysis.

D. Enzyme Assay

The crude enzyme extract was assayed for cellulase (CMCase) activity by Dintrosalicylic method [16]. The endoglucanase activity was determined according to the method given in [17] wherein which the reaction mixture containing 0.5 ml crude enzyme and 0.5 ml substrate was incubated at 50°C for 30 minutes. Substrate for assay was prepared by dissolving 1% (w/v) carboxymethylcellulose (High viscosity, HiMedia) in 0.1 M phosphate buffer (pH 7). The reaction was stopped by adding 1.5 ml DNS and further boiled at 100°C for 5 minutes followed by cooling with water for colour stabilization. The amount of reducing sugars released during the assay was determined by measuring the absorbance at 560 nm using spectrophotometer (Labindia). CMCase activity was calculated by using glucose calibration curve. One unit (IU) of CMCase activity was described as the amount of CMCase needed to release 1 micromole of reducing sugars per ml per minute under the given assay conditions.

E. Molecular Identification by 16S rRNA Gene Sequencing

The genomic DNA from pure culture of the selected isolate was extracted using HiPurA^{TM} Plant Genomic DNA Miniprep Purification Spin Kit (HiMedia) as per the manufacturer's instructions. The amplification of 16S rRNA gene was carried out using the following primers: 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (3'-ACG GCT ACC TTG TTA CGA CTT-5') [18]. The sequencing of the purified 16S rRNA gene was performed using four sequencing primers and Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA). PCR reaction mixture was comprised of 200 µM dNTPs (Fermentas, Vilnius, Lithuania), 50 pmol each primer, 1× PCR buffer, 1 U Taq DNA polymerase (Promega, WI, USA), and 100 ng genomic DNA. The thermocycling procedure involved an initial denaturation at 94°C for 4 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min, and final extension at 72°C for 8 min.

F. Sequence Assembly and Phylogenetic Analysis

The sequence data obtained were assembled and further analyzed using DNA sequence assembling software SEQUENCHER™ 4.10.1 (Gene Codes Corporation, USA). The sequences were used as query to blast in order to find the similar sequences in nucleotide database of NCBI (http://www.ncbi.nlm.nib.gov/). Phylogenetic tree was constructed after aligning all acquired and related sequences with Clustal W software [19], using the neighbour-joining method in MEGA 4 and the Kimura 2 parameter model, and bootstrapped with 1000 replications [20].

G. Optimization of Culturing Conditions for Cellulase Production

Growth conditions like incubation time, temperature, carbon source and substrate concentration were optimized by taking one-factor-at-a-time approach. A single colony of the bacterial isolate was inoculated into 100 ml Erlenmeyer flask containing 20 ml nutrient broth and incubated at 37°C for 24 hours. This culture served as the source of primary inoculum for further secondary inoculation.

1). Incubation Time: For optimizing incubation time for enzyme production, 100 ml nutrient broth media in a 250 ml

Erlenmeyer flask supplemented with 1% (w/v) wheat bran as the carbon source was prepared. Secondary inoculation of the sterilized media was carried out with 1% (v/v) primary culture and incubated in an incubator-shaker at 37°C. Enzyme extraction was performed by centrifugation at regular intervals of time (6, 12, 24, 48, 72, 96 and 120 hours) and enzyme assays were performed.

2). Carbon source: Erlenmeyer flasks with 100 ml nutrient broth medium were prepared supplemented with 1% (w/v) of the following carbon sources: glucose, lactose, sucrose, carboxymethylcellulose (CMC), rice bran and wheat bran. All the media flasks were inoculated with 1ml of primary culture and incubated further at 37°C for 72 hours in an incubator-shaker with 150 rpm agitation speed.

3). Incubation temperature: 100 ml nutrient broth medium supplemented with 1% wheat bran was prepared and sterilized. The media was inoculated and incubated at different temperatures for 72 hrs with agitation at 150 rpm. To study the temperature effect, different temperatures 28°C, 30°C, 37°C and 45°C were employed to find out the optimal temperature condition for CMCase production.

4). Substrate concentration: Different concentration of wheat bran like 1%, 2%, 3%, 4% and 5% were used in 100ml production media (nutrient broth). The flasks with different combinations were inoculated and incubated at 30°C, 150 rpm for 72 hrs. Enzyme assays were performed by using DNS method to determine effect of these conditions on cellulase production.

III. RESULTS

A. Isolation and Screening of Cellulose Degrading Bacteria

Seventy bacterial isolates in total were isolated from the various soil samples which were collected from different agricultural fields. Out of these, 16 were able to degrade cellulose and these isolates were screened on the basis of Congo red assay. The hydrolysis capacity of all the isolates was calculated (Table I).

Gram staining of the isolates revealed that out of the 16 isolates, 15 were found to be Gram-positive and 2 were Gram-negative when examined with microscope after Gram staining. All the isolates had rod like morphology. Some were present as isolated rods and other were in the form of chains. Morphological examination of the isolates with cellulose degrading ability was displayed in the Table II.

TABLE I
Hydrolysis Capacity of Different Bacterial Isolates

S. No.	Isolate	Hydrolysing capacity (HC)
1.	S 4	0.39
2.	S 9	1.2
3.	S 10	2.0
4.	S 13	2.1
6.	S 18	1.67
7.	S19	0.6
8.	S 21	0.9
9.	S 22	1.5
10.	S 23	2.0
11.	S 24	1.9
12.	S 25	1.1
13.	S 26	1.4
14.	S 27	0.6
15.	S 42	1.9
16.	S 45	2.1

TABLE II
Morphological and Gram Staining Characteristics of Different Isolates

S. No.	Isolate	Colony characteristics (color, shape, texture, diameter)	Gram character and morphology
1.	S 4	Yellowish, entire, smooth, glistening, raised colonies	Gram positive rods isolated
2.	S 9	Creamish white, undulate, rough, opaque, flat colonies	Gram positive rods in chains
3.	S 10	Creamish white, entire, smooth, opaque, raised, mucoid colonies	Gram positive rods in chains
4.	S 13	White, entire, smooth, opaque, flat colonies	Gram positive isolated rods
5.	S 14	Creamish, entire, smooth, opaque, flat colonies	Gram positive isolated rods
6.	S 18	Yellowish, entire, smooth, glistening, raised colonies	Gram positive rods in chains
7.	S 19	Creamish, entire, smooth, glistening, raised colonies	Gram negative rods in chains
8.	S 21	Creamish, irregular, rough, flat colonies	Gram positive isolated rods
9.	S 22	Yellowish, irregular, opaque, raised colonies	Gram positive isolated rods
10.	S 23	Yellowish, entire, smooth, opaque, convex colonies	Gram negative isolated rods
11.	S 24	Creamish white, entire, rough, opaque, umbonate colonies	Gram positive rods in chains
12.	S 25	Creamish white, undulate, rough, opaque, flat colonies	Gram positive small rods in chains
13.	S 26	Yellowish irregular, dull, opaque, raised colonies	Gram positive rods isolated
14.	S 27	Yellowish, entire, smooth, glistening,	Gram positive rods in chains

		punctiform, flat colonies	
15.	S 42	Yellowish, entire, smooth, glistening, opaque, flat, punctiform colonies	Gram positive very small rods in chains
16.	S 45	Creamish, entire, opaque, glistening, raised colonies	Gram positive long rods

B. Enzyme Production by Submerged Fermentation

All the isolates were grown in liquid medium and incubated for 48 hrs at 37°C with 150 rpm agitation speed. The cells were harvested by centrifugation and supernatant was assayed for determination of cellulose-degrading activity (Table III). All the isolates exhibited cellulose-hydrolysing activity ranging from 0.19 to 0.92 IU/ml. The maximum enzyme activity was shown by the isolate S 18. This isolate was selected to carry out further studies.

Morphological examination of the bacterial strain revealed yellowish, entire, smooth, glistening, raised colonies. The clearance zone shown by bacterial isolate no. 18 after the Congo red assay was displayed in Fig. 1. Bacterial strain was Gram positive and rods in chains were seen microscopically after Gram's reaction. The EZTAXON analysis of 16S rRNA gene sequence of the strain showed 99.9% identity with *Bacillus subtilis* subsp. *inaquosorum* BGSC 3A28T. The nucleotide sequence obtained after sequencing was submitted in GENBANK under the Accession no. KP765601 (Table IV). Phylogenetic analysis was carried out and tree was constructed by the Neighbor joining method (Fig. 2). Therefore, isolate S 18 was identified as *Bacillus subtilis* subsp. *inaquosorum.*

4.	S 13	0.50
5.	S 14	0.70
6.	S 18	0.92
7.	S 19	0.69
8.	S 21	0.35
9.	S 22	0.11
10.	S 23	0.65
11.	S 24	0.76
12.	S 25	0.83
13.	S 26	0.22
14.	S 27	0.39
16.	S 42	0.82
17.	S 45	0.61

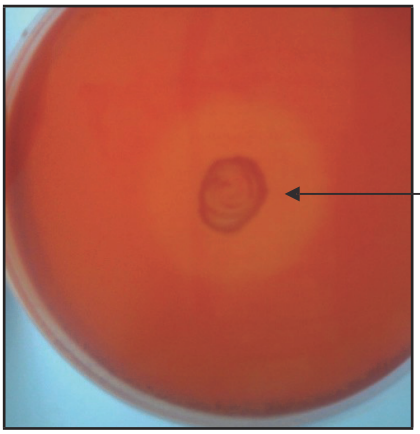

Fig. 1 Bacterial isolate showing CMCase activity after Congo red assay

TABLE III
Cellulase Activity of Different Bacterial Isolates

S. No.	Isolate	Cellulase activity (IU/ml)
1.	S 4	0.25
2.	S 9	0.41
3.	S 10	0.19

TABLE IV
Cellulase Activity of Different Bacterial Isolates

Isolate No.	Organism (Closest match)	Sequence length (bp)	Accession no.	Identity (%)
S 18	*Bacillus subtilis* subsp. *inaquosorum* BGSC 3A28T	1,515	KP765601	99.9

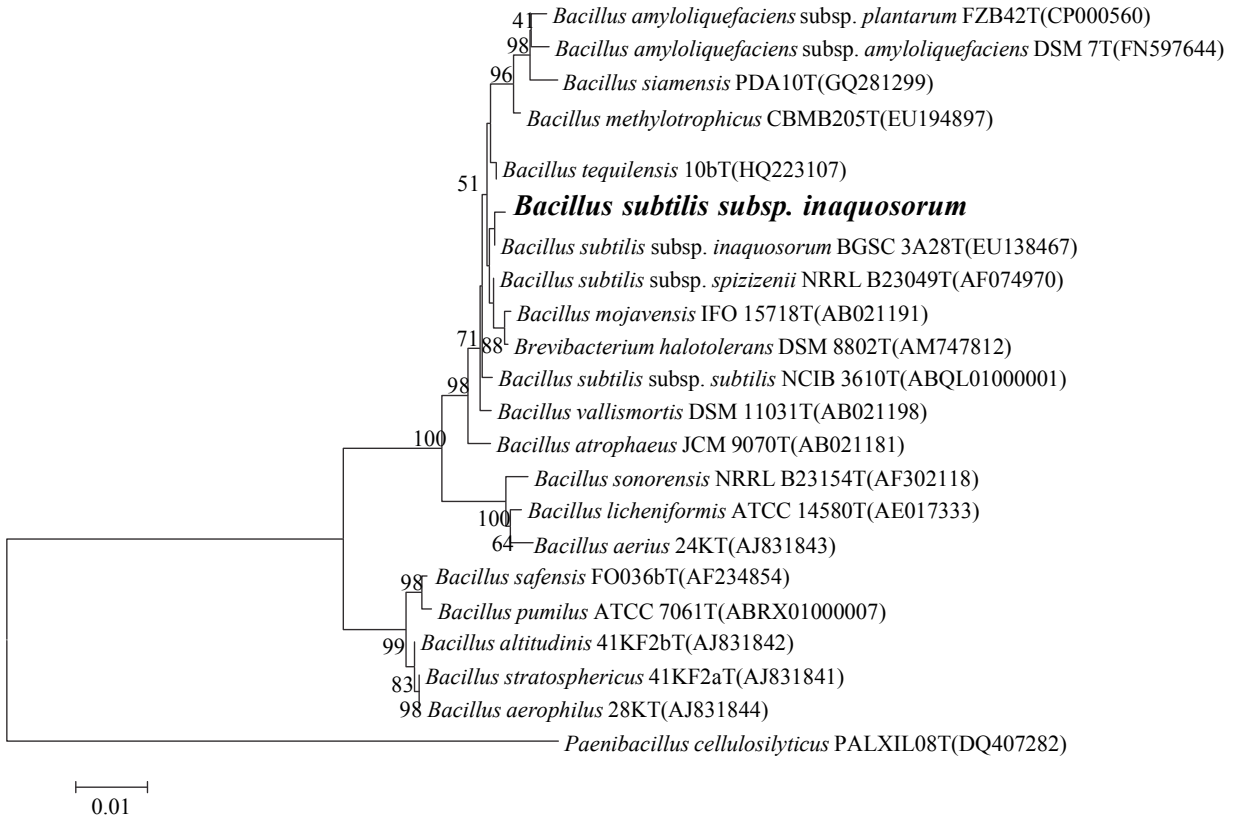

Fig. 2 Phylogenetic tree based on 16S rRNA gene sequence of bacteria constructed using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and were conducted in MEGA4.

C. Optimization of the cultural conditions

To find out optimal incubation time for the cellulase production by *B. subtilis* subsp. *inaquosorum,* the production medium was inoculated with 1% inoculum and incubated at 37°C with orbital shaking (150 rpm). The cellulase activity was determined at various time intervals till 120 hrs. CMCase activity increased with time and maximum production (1.15 IU/ml) was obtained after 72hrs of incubation after which it decreased rapidly. CMCase production increased with increase in biomass but maximum production was observed in the stationary phase (Fig. 3).

Effect of temperature on CMCase activity was studied by incubating liquid media (NB + 1% Wheat bran) inoculated with *B. subtilis* subsp. *inaquosorum*, at different temperatures for 72 hrs under shaking conditions (150 rpm). The study revealed that the microbe grew well at incubation temperatures 25°C, 30°C and 35°C and produced appreciable cellulase activity (0.9-1.15 U/ml). At 40°C and 45°C, cellulase activity decreased to 0.45 and 0.12 U/ml respectively. The optimum incubation temperature recorded for cellulase activity (1.15 U/ml) was 30°C, suggesting that *B. subtilis* subsp. *inaquosorum* was mesophilic in nature (Fig. 4).

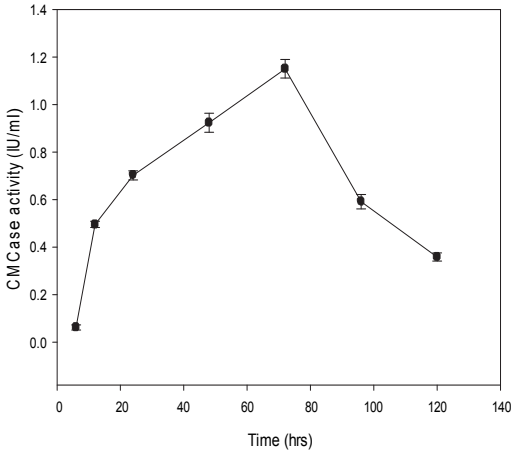

Fig. 3 CMCase activity with change in incubation time. Values were expressed as mean ±S.D. of three individual values.

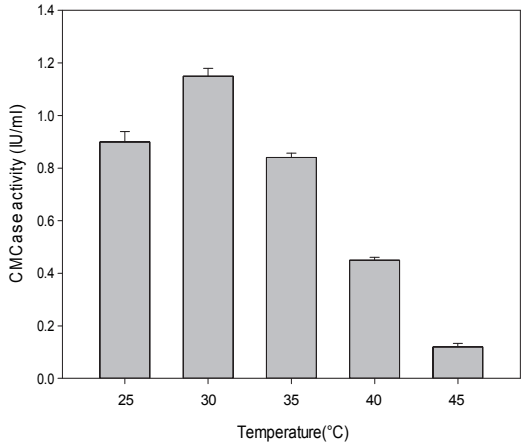

Fig. 4 CMCase activity with change in incubation temperature. Values were expressed as mean ±S.D. of three individual values.

Bacillus subtilis subsp. *inaquosorum* was subjected to submerged fermentation and effect of various carbon sources (1%, w/v) on CMCase activity was tested. Presence of simple sugars such as glucose, lactose and sucrose, in NB supported excellent growth of *B. subtilis* subsp. *inaquosorum*, but cellulase activity was diminished. Maximum CMCase activity (1.29 IU/ml) was observed with wheat bran as carbon source (Fig. 5).

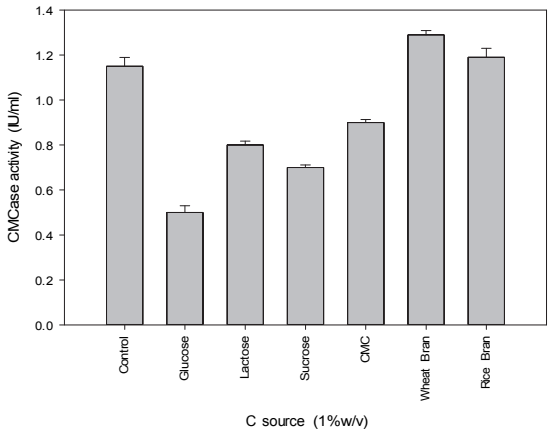

Fig. 5 CMCase activity with different carbon sources. Values were expressed as mean ±S.D. of three individual values.

NB media supplemented with different concentration (1-5% w/v) of wheat bran as carbon source was inoculated with *B. subtilis* subsp. *inaquosorum* and incubated at 37°C for 72 hrs under shaking conditions (150 rpm). Cellulase activity was determined and maximum activity (1.38 IU/ml) was observed with 3% substrate concentration of wheat bran as depicted in Fig. 6.

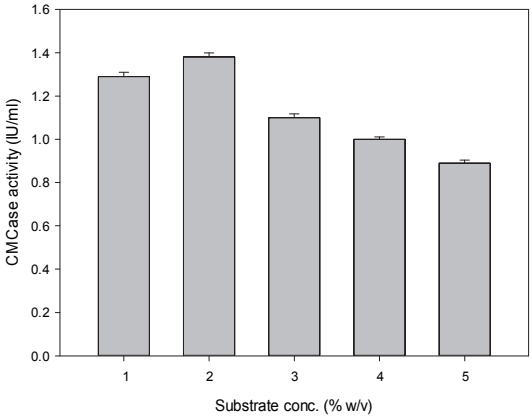

Fig. 6 CMCase activity with different carbon sources. Values were expressed as mean ±S.D. of three individual values.

IV. DISCUSSION

Numerous enzymes have established themselves as potential industrial candidates, till date. But still the present candidates are not enough to fulfill all the requirements at industrial scale. The high cost of enzyme is the major hurdle in the path of its commercialization. At present, enzymes with high titers and good stability over broad range of conditions are needed to utilize them in various biotechnological applications. Cellulase market is gaining momentum as they are considered as biotechnological potential candidates exploitative in various industrial applications. Cellulases are finding applications in various commercial sectors viz. pulp and paper industry; textile industry; food and feed industry; pharmaceutical industry and biorefinery etc. [13], [14]. Considering the demand for cellulases at commercial scale, this study has been carried out to optimize conditions for enhanced production of CMCase from *B. subtilis* subsp. *inaquosorum*.

Although cellulase activity of bacteria are usually observed to be less than the fungal members but bacteria are gaining much attention as cellulase producers due to their occurrence in wide variety of natural habitats thereby possessing the ability to withstand extreme environmental conditions. In addition, bacteria have higher growth rate and produce enzyme complements which are stable at extreme temperature and pH. They are less affected by feedback inhibition (end product accumulation) during the cellulose hydrolysis. They can be genetically engineered easily in comparison to fungal cellulases [21]-[23]. Literature search clearly indicate that *Bacillus* spp. are good producers of extracellular enzymes [13], [24]-[26].

The yield of cellulases produced by different microorganisms is remarkably affected by various parameters like pH, temperature, aeration, incubation time and growth nutrients [27], [28]. Therefore, evaluation of various nutritional and environmental factors for enzyme

production plays a pivotal role in success of a process technology. Wheat bran was found to be the best source yielding maximum amount of CMCase. Numerous studies have reported similar results where wheat bran was found to be the inducer for cellulase production [29]-[31]. Temperature is considered as an important factor which significantly affects the enzyme production. Similar results were reported for *B. subtilis* [25] and *Pseudomonas* sp. [32] where 30^0C was found to be the optimum temperature for cellulase production. *Psuedomonas* sp. HP207 has been reported to have cellulolytic activity of 1.432 U/ml with yeast extract, bean flour and ammonium chloride observed as favourable media components for its production [32]. Similarly, maximal cellulase production was reported up to 0.38 IU/ml at pH 7 of the media after incubation at 37^0C [33].

Over the years, bacteria are gaining attention due to their several advantages in cellulosic biomass hydrolysis. In this study, cellulose hydrolyzing bacteria *B. subtilis* subsp. *inaquosorum* was isolated from soil and optimization of the cultural conditions was carried out to enhance CMCase production. Further, statistical studies and genetic engineering of the enzyme can be employed to study its biochemical properties in order to introduce this enzyme in the commercial sector.

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