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Isolation, Identification and Characterization of Cellulolytic Bacteria from Soil in Peshawar Region Pakistan

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Abstract

We isolated three cellulose degrading bacteria based on clear zone using Congo red stain on carboxymethyl cellulose (CMC) agar plates. Three bacterial isolates named as HB2, HS5 and HS9 which were subsequently characterized by 16S rRNA gene sequencing, morphological and biochemical tests. Based on 16S rRNA analysis, the bacterial isolates were identified as Bacillus *cerus, Bacillus subtilis* and *Bacillus altitudinis*. For maximum cellulose enzyme production, different growth parameters were optimized. HB2 showed maximum optical density for growth at 40 °C for 72h while HS5 and HS9 at 40°C for 48 h. Maximum optical density for growth was also noted at pH 7 for 48 h for all three isolates. Optical density was high for all three isolates using meat extract as a nitrogen source for 48 h. The pH profile of all three strains was quite similar but the maximum enzyme activity was observed at pH 7. Maximum cellulose production was noted by all three bacterial isolates using lactose as a carbon rather than nitrogen and peptone.

Further studies are needed in region for identification of new isolates having maximum cellulolytic activity. Above finding indicates that this enzyme can be potentially used in various industrial applications.

Keywords: Cellulose; Cellulose Enzyme assay; Carboxymethyl Cellulose

Abbreviations: BLAST (Basic Local Alignment Search Tool); CMC (Carboxymethyl cellulose); DNS (Dinitro salicylic Acid); EMB (Eosin methylene blue); pH (Power of hydrogen ion); MEGA (Molecular Evolutionary Genetics Analysis); MSA (Mannitol salt agar); nm (Nanometer); NCBI (National Center for Biotechnology Information); OD (Optical Density); Rpm (Revolutions per minute); rRNA (Ribosomal RNA)

Introduction

Most of the biomass present on earth is in the form of cellulose which is major constituent of cell wall and accounts 50% for dry weight [1]. In plant biomass, Cellulose accounts for 40 billion tons per year, which makes it the most abundant and primary product [2]. Plant biomass is also present in less amount in hemicelluloses and least of all lignin [3]. Microorganisms that are found in nature like bacteria and fungi, both has the ability to degrade different types of cellulose which can be soluble (amorphous) and insoluble (crystalline) by the action of celluloses and hemicelluloses [4]. In plant biomass, structural polysaccharides can be degraded by combined effects of enzymes such as celluloses, hemicelluloses, and glycosyl hydrolases. Cellulose play important role in the breakdown of complex structure into simple monomeric form which are industrially applicable [5]. Cellulose found in the cell wall of plant is commonly degraded by the combined action of a multi-component enzymatic system of cellulose which play main role in bioconversion of cellulosic material [6]. The hydrolysis of cellulosic biomass is performed by synergistic action of three main enzymes. These enzymes are cellobiohydrolase or exoglucanase, endoglucanase or carboxymethyl cellulaose and cellobiase or b-glucosidase [7].

Bacteria can be used to degrade cellulose into simple sugars because they can adapt to environmental conditions and biochemical changes such as *Pseudomonas aeruginosa*, *Serretiamarcescens*, *Nocardia*, *Arthrobacter*, *Micrococcus* etc. Bacterial studies are of great importance due to their cellulolytic enzyme production and biochemical flexibility [8]. Many bacterial strains have been discovered from soil that has ability to degrade cellulosic biomass. [7]. Keeping in view the potential applications of cellulose enzyme in different industrial processes, it is necessary to isolate, identify and characterize cellulose producing bacteria from soil. In this study, three bacterial strains were isolated from soil of bagasse of sugar mill and sawdust from carpenter shop and were screened for their cellulolytic activity. These bacterial strains were identified by 16S rRNA gene sequencing and different culture parameters have been examined for enzyme production such as time, temperature, pH, and nitrogen and carbon sources. Furthermore, also the operational stability has been investigated to full fill the requirement of different industrial processes.

Materials and Methods

Isolation of cellulolytic bacteria from soil samples

Cellulolytic bacteria were isolated from different soil samples collected aseptically near Khazana Sugar Mills, Peshawar. The Soil samples were collected, and isolation of cellulolytic bacteria was done by serial dilution technique up to 10^{-6}. Samples were spread on CMC Nutrient agar supplemented with Congo red. The positive isolates were subsequently sub-cultured and further identified through morphological and biochemical tests.

Identification of selected isolates

The selected bacterial isolate that showed the highest cellulose activity was identified based on the morphological and biochemical parameters.

Molecular identification of bacterial isolates

Pure culture of positive bacterial isolates was sent to Macrogen Korea Sequencing Company for DNA extraction and subsequent 16s rRNA gene sequencing using universal primers (Lane, 1991). The amplified 16s rRNA PCR product was sequenced and trimmed. The unknown organism was identified using the maximum aligned 16s rRNA sequences available in the GenBank of NCBI through BLAST search. MEGA 6.0 software was used for the construction of phylogenetic tree to understand the evolutionary relationship (Tamura *et al.*, 2007).

Secondary screening for Production of Cellulose Enzyme

The isolates showed maximum cellulolytic activity were subjected to enzyme production. A single colony was used as inoculum source for the production medium [9]. In 50 ml of Erlenmeyer flasks, 25 ml of nutrient broth containing 1 % carboxy methyl cellulose (CMC) was prepared for each of the selected isolate's strains including a control and autoclaved at 121°C for 15 minutes. Broth media was inoculated with 250 µl of bacterial culture and incubated overnight at 37°C for 150 rpm to obtain clear supernatant. The fermented broth was centrifuged at 10,000 rpm for 10 mins and supernatant was used for enzyme activity assay [3].

Enzyme Assay

Enzyme assay was carried out according to the method described by Kumar et al. (2012). 100 µl of crude enzyme supernatant along with 100 µl of 1% CMC as a substrate and cellulose activity was measured by estimation of glucose by DNSA method. One unit of cellulose activity is defined as ″the amount of cellulose required to catalyze the formation of reducing sugar which is equal to 1 mole of D glucose per minute under assay conditions″ [10] [11].

Enzyme activity (U/ml) =
Reducing sugar (product concentration) X 1000 X dilution factor
Molecular weight of glucose X incubation time (minute)

Enzyme production under Optimized Conditions

Optimization of the fermentation medium for maximum cellulose production was carried out. The effect of various factors affecting cellulose production was determined by measuring enzyme activity at different pH value (5 - 9) and temperature (35-50 °C) [12] [13]. The effect of various carbon sources such as maltose, lactose, and fructose concentration were examined in the production medium. Various nitrogen sources like yeast extract, peptone and meat extracts were examined for their effect on enzyme production and growth of bacterial isolates.

Results

Screening and Identification of Cellulolytic Bacteria

Three cellulose positive isolates were observed on CMC agar plates supplemented with Congo red which showed zones of clearance on adding Gram's iodine indicating production and release in the CMC agar plates (Supplementary Figure 1). These three isolates were named as HB2, HS5 and HS9 (Table 1). The cultural, biochemical, and morphological studies were carried out and it was seen that all isolates were gram positive in nature (Supplementary Table 1).

Molecular identification of Cellulolytic Bacteria

Pure culture of positive isolates was sent to Macrogen Korea company for 16S rRNA gene sequencing using universal primers (Supplementary Table 1) [14]. 16S rRNA gene sequencing revealed the presence of three positive bacterial isolates include: B*acillus cerus, Bacillus subtilis* and *Bacillus altitudinis*. The phylogenetic tree was constructed for the identified bacterial isolates through MEGA 6.0 to understand evolutionary relationship (Supplementary Figure 2, 3, and 4) [15].

Sample ID	Citrate Test	Eosin Methylene Blue	Mackon key	Blood Agar	MSA Media	Coagulase Test	Oxidase Test	Urease Test	Catalase Test	Indole Test	Identified Genus
HB2	Negative	Negative	Pink growth	Greenish growth	Negative	Negative	Negative	Negative (yellowish color)	Positive	Negative	Bacillus
HS5	Negative	Purple growth	Pink growth	Greenish Growth	Yellow growth	Negative	Positive	Medium positive	Positive	Negative	Bacillus
HS9	Negative	Negative	Negative	Greenish growth	Yellow growth	Positive	Positive	Slow positive	Positive	Negative	Bacillus

Table 1: Biochemical Characteristics of Bacterial Isolates

Effect of Temperature and Incubation Time on Enzyme Production

HB2 showed maximum cellulose production and optical density activity for 72 h at 40°C. (Figure 1a). The optimum cellulose activity of HS5 was observed for 24 h at 40°C for 24 hours. The maximum activity of HS9 at 40°C for 48 h and the maximum optical density was at peak at 40°C for 48 h measured at 600 nm (Figure 1b and 1c).

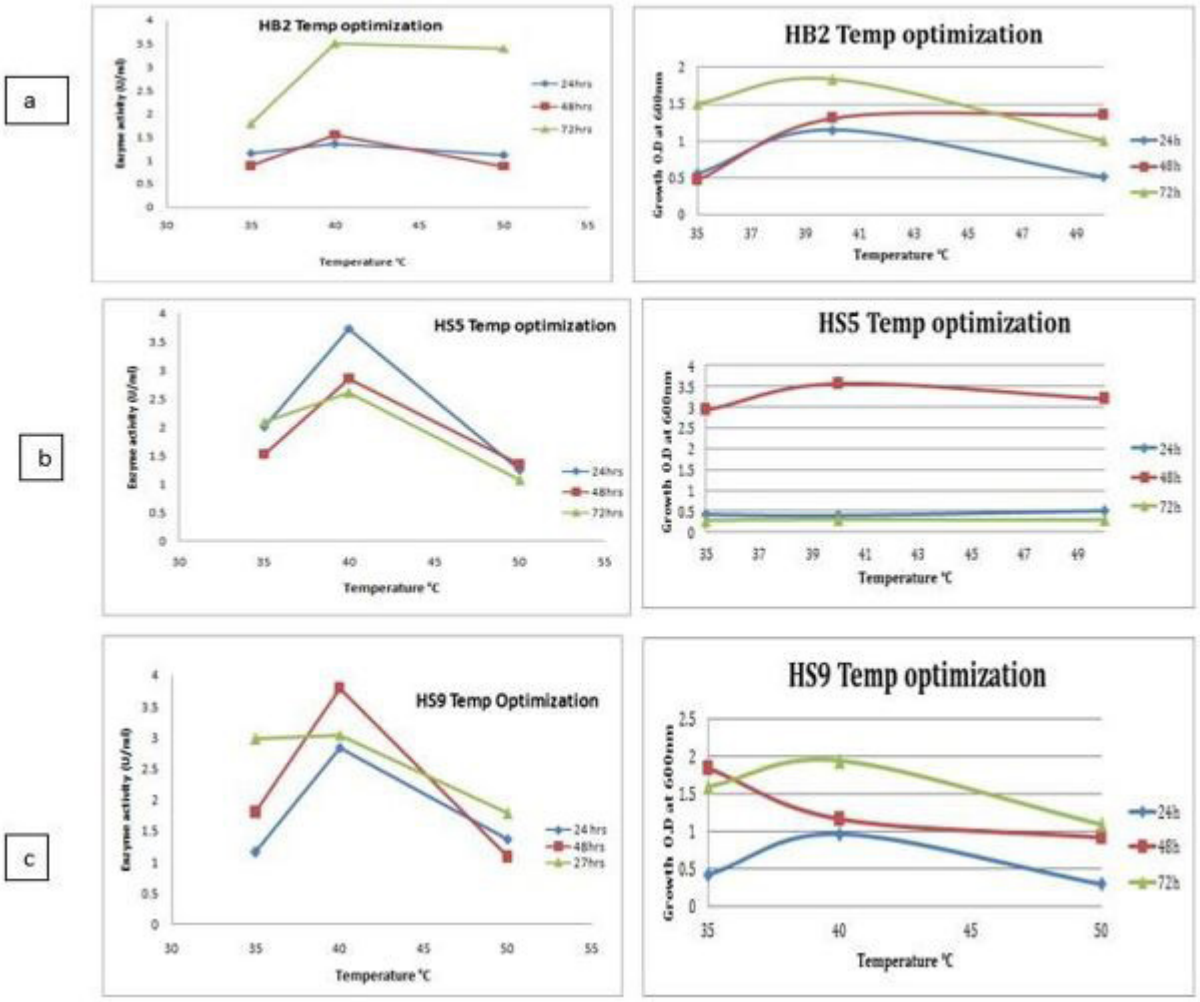

Figure 1: Effect of incubation temperature and time on enzyme activity and growth of (a) HB2 (b)HS5 and (c) HS9

Effect of pH on Enzyme Production

The growth medium pH is one of the most important physical parameters which played an important role in enzyme secretion. The fermentation was carried out with CMC in shaking incubator for 72 hat pH ranged 3-5 to determine optimum pH for enzyme production. The best optimum cellulose production was noted at pH 7 for 48 hours for HB2 and optimum growth density was at peak at pH for 48 h (Figure 2a). The maximum cellulose production and optical growth density of HS5 at pH 7 for 48 h are presented in Figure 2b. Maximum enzyme production for HS9 was observed at pH 7 for 24 h with maximum optical growth density at pH 7 for 48 h (Figure 2c).

Effect of Carbon Source on Enzyme Production

The cellulolytic bacteria were treated with different carbon sources (fructose, maltose, and lactose) at 1% concentration at different incubation time interval (24h-72h). The maximum enzyme activity and optical density of growth was observed for HB2 using lactose as a carbon source for 48 h time interval (Figure 3a). Whereas HS5 also showed maximum production of enzyme and optical density for 24 h using lactose as a carbon source. Lactose was found to be the best source of carbon for the bacterial isolate HS9 in the production of cellulose enzyme for 48 h time interval, while the optical density for growth of HS9 was also on peak for fructose as a carbon source for 48 h interval period. (Figure 3b and 3c).

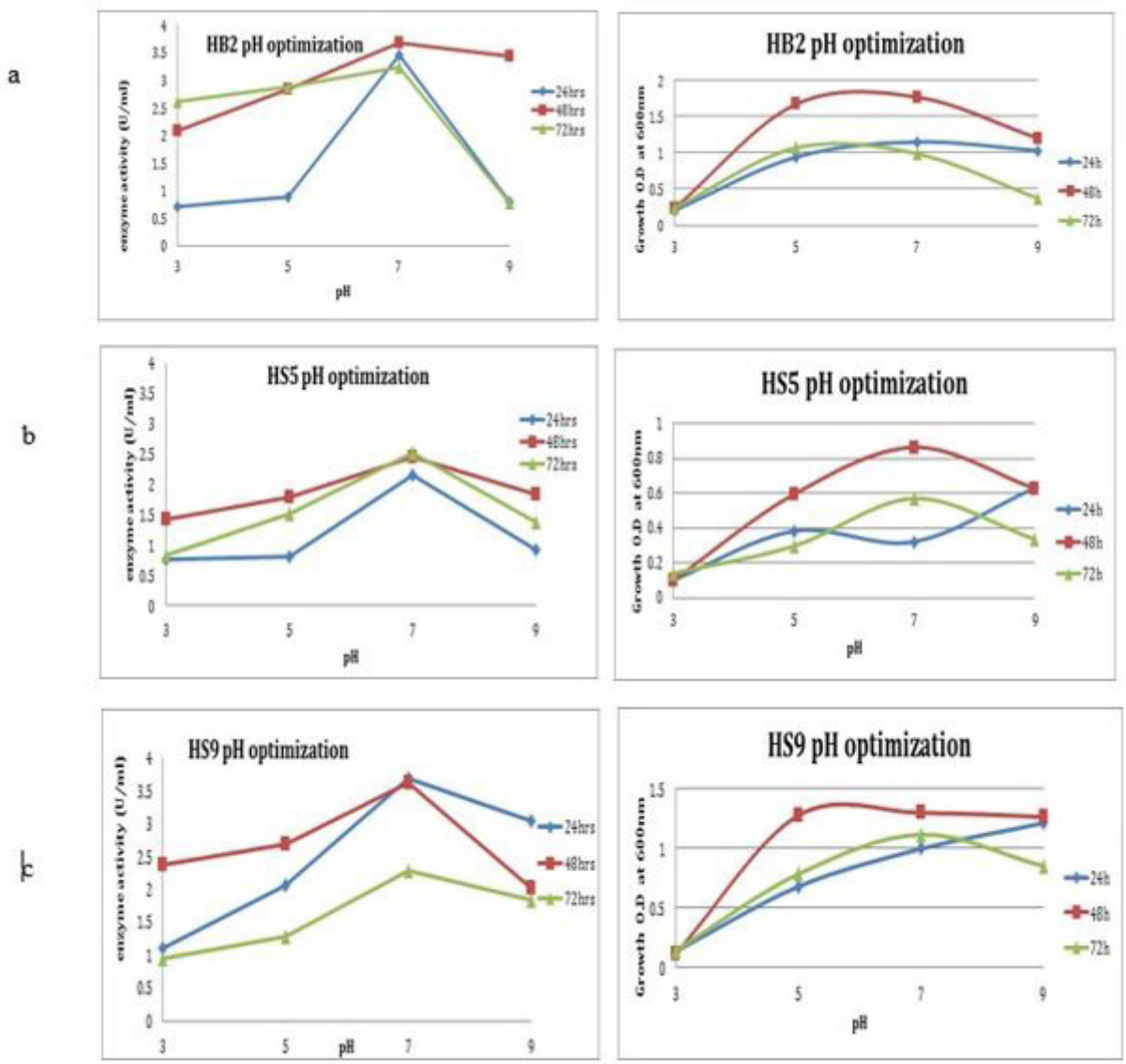

Figure 2: Effect of pH on cellulose enzyme production and growth of (a) HB2 (b) HS5 and (c) HS9

Effect of Nitrogen Sources on Enzyme Production

Nitrogen is an essential element that play important role in the growth of microorganism and in the production of enzymes. Different nitrogen sources were used (Meat extract, Yeast extract, and Peptone) at concentration of 1%. The bacterial strain HB2 showed optimum enzyme production while optical density was at peak using meat extract as a nitrogen source at 48 h interval (Figure 4a). The maximum cellulose production was noted by HS5 for 24 h but the optical density was at peak for HS9 at 48 h interval time using meat extract as a nitrogen source (Figure 4b). Maximum enzyme activity was also noted by bacterial strain for 72 h using peptone as a nitrogen source. Although, HS9 revealed maximum optical density for growth at 48 h interval when peptone was used as a nitrogen source. (Figure 4c).

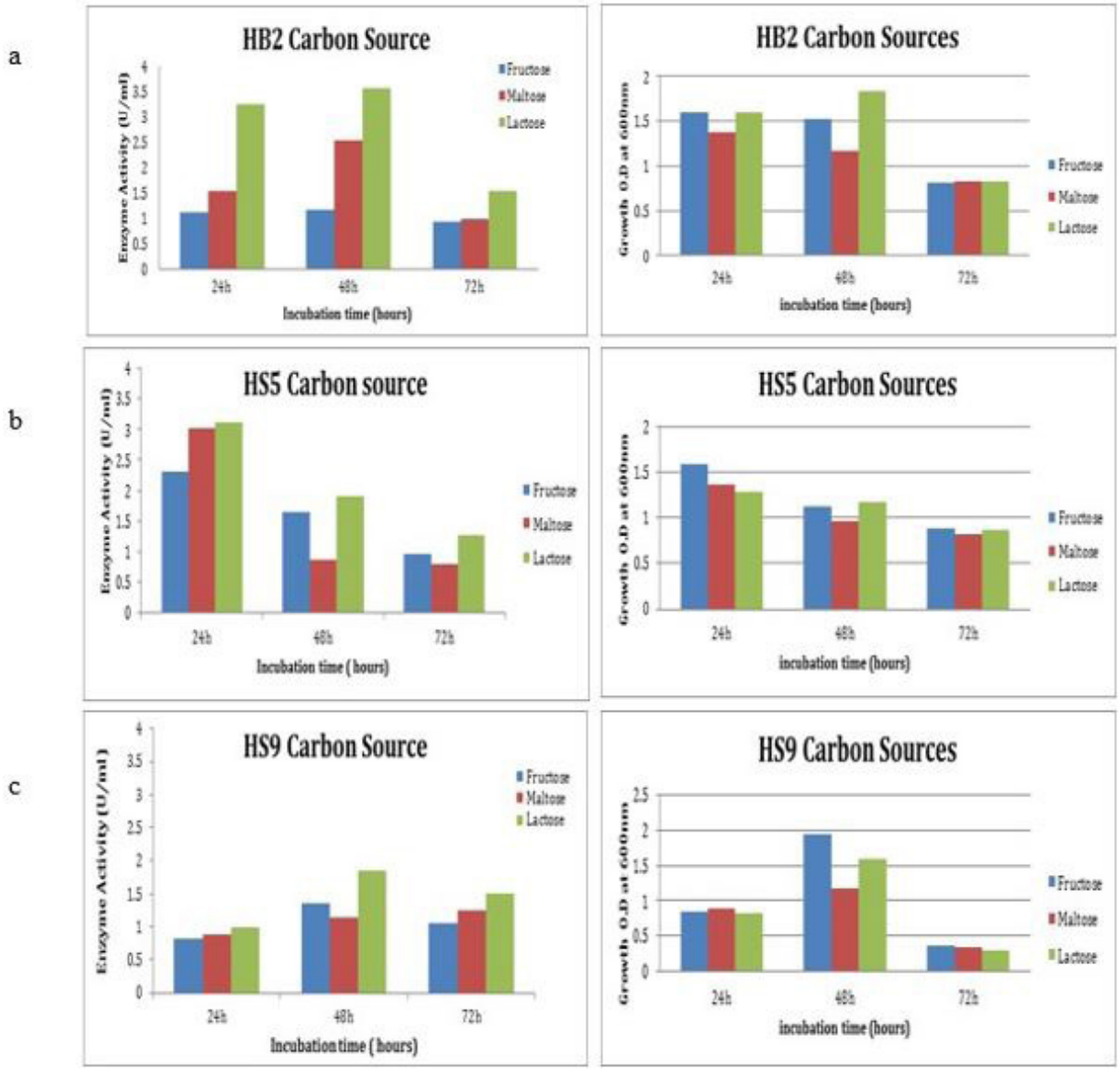

Figure 3: Effect of carbon sources on enzyme activity and growth of (a) HB2 (b) HS5 and (c) HS9

Discussion

The natural environment is full of biological waste that can be used by the microorganism for beneficial purposes. Those microorganisms having the ability to degrade cellulose to form cellulose. In most of the reference studies, cellulolytic bacteria were isolated from different natural environments [16] whereas in the current study, cellulolytic bacterial strains were also isolated from natural environment. In most of the reference studies, the organisms isolated from the natural environment belonged to *bacillus* species which were capable of degrading cellulose producing cellulose enzymes. The aim of the present study was to demonstrate the isolation, identify bacteria with high cellulose activity from soil samples. In our research, we isolated three cellulose producing bacteria from natural environment and were analyzed through 16S rRNA gene sequencing and identified as B*acillus cerus, Bacillus subtilis* and *Bacillus altitudinis*. Furthermore, the phylogenetic analysis revealed the evolutionary relationship.

Celluloses producing bacterial strains were identified on their ability to produce clear zone on CMC agar plate. The biochemical characteristics of the isolates were found to be positive for the bacterial isolates by performing several biochemical tests like Fermentation test, Catalase test, Citrate utilization test, Methyl-red test, H2S production and Voges Proskauer test by standard methods. Different growth parameters such as pH, temperature, incubation period, substrate concentration and carbon sources were optimized for enzyme production [17]. Soil is one of the main

reservoirs which provide nutrient and energy for supporting the growth of microorganisms. *Bacillus cerus* and *bacillus subtilis* is the most common organism found in soil. In the reference study, novel bacterial strain *bacillus altitudinis*, was 1st isolated from high attitude sample collecting cryogenic tubes [18]. However, *bacillus altitudinis* was also reported in the spoilage and soft rot of vegetables and fruits in the study conducted in Egypt [19]. In the current study, the samples were taken from saw dust and the bacterial strain isolated and identified using 16s rRNA as *bacillus altiduinis*, which is very rare to identified it from soil.

Cellulose producing microbes were found to have optimum cellulose activity of temperature ranging from 30-50°C. The effect of temperature for enzyme activity is also an important step for production of enzyme [20] [21]. In the present study, the optimum temperature to produce enzyme using CMC in the concentration of 1% in the flask was also at 40°C. There was a significant amount of increase in the amount of enzyme production was observed at 40°C temperature [22]. All three isolates were allowed to grow at different pH ranging from (3, 5, 7, and 9) and the maximum activity was observed at pH 7 by all bacterial isolates (Figure 4). This study was in correlation with the finding of other works for different *Bacillus* strains [23].

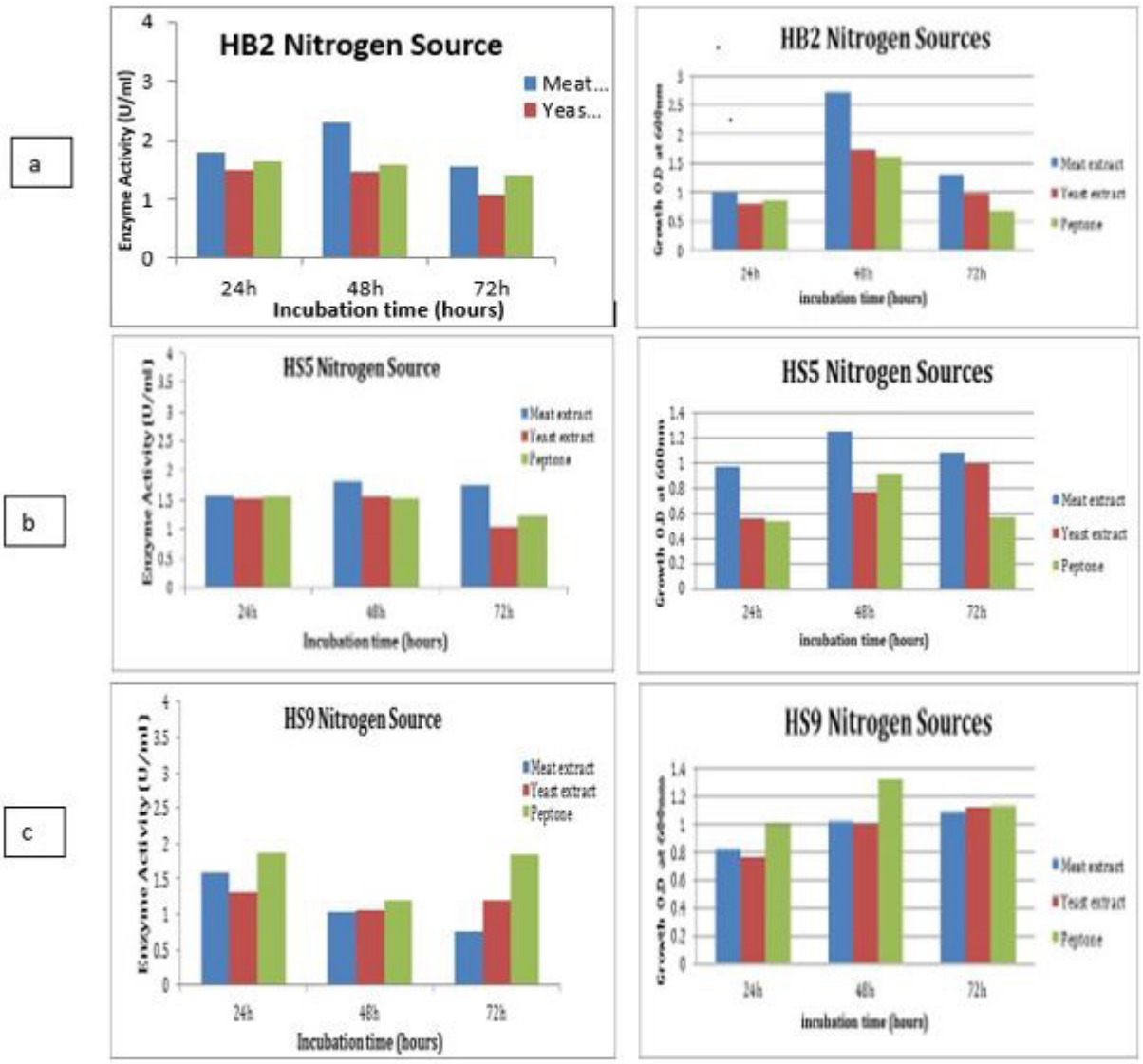

Figure 4: Effect of nitrogen sources on cellulose enzyme activity and growth of (a) HB2 (b) HB5 and (c) HB9

The maximum enzyme activity and optical density of growth was observed for HB2 using lactose as a carbon source for 48 h time interval. While maximum cellulose activity was also observed by HS5 for 48 h time interval suing meat extract and peptone as a nitrogen source. In the reference study, the maximum cellulose production was optimized at 25°C using lactose as a carbon source and meat extract was used as a nitrogen source which support our current findings [24]. The same results were also observed by Gohel et al, 2014. on CMC agar plates [25]. The maximum production of cellulose was also observed by utilizing peptone by bacillus specie K1 peptone [26]. Which are consistent with our current findings. In conclusion, all the three bacterial isolates showed maximum cellulose enzyme production activity for meat extract as nitrogen source and lactose as carbon source (Figure 3 and 4).

Conclusion

In this study, three different cellulolytic bacteria were isolated from natural environment such as *Bacillus cereus*, *Bacillus subtilis* and *Bacillus altitudinis*. Different growth parameters were optimized that improved performance of cellulose producing bacteria and stability of cellulose. In the current study, we find out that when lactose was used as a carbon source, meat extract as a nitrogen source, the selected isolates showed maximum enzyme activity at pH 7 and at 40°C, so far, the *Bacillus altitudinis* has been reported from high altitudes, but in this study, we reported it from a carpenter shop at Board bazar. Based on the result of the current study it is recommended that in future, on large scale it should be executed to find out enzyme activity and its production at industrial scale.

Limitations

Although the parameters are optimized for maximum production of cellulose at lab scale but for industrial-level production, the respective parameters should be assessed thoroughly at large scale. Besides the isolates identified in the current study, this region may have other bacteria that have better enzyme production capacity which needs to be isolated, characterized and conditions may be optimized at small and then at large scale to boost the industry. This will also reduce the production cost ultimately benefiting the consumers at the national and international levels.

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Author's contribution

Hira Ikram performed experiments, data analysis and manuscript writing, Hina Ali, Yaseen Ahmad and Jawairia Kiran carried out bioinformatics assistance and organizing the data, Hamid Ali Khan conceived and designed the study. All authors have read and approved the final manuscript.

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Availability of data and material

All the data generated or analyzed during this study are included in the manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

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