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Full Length Research Article

Isolation, Screening and Media Optimization of Cellulase Producing Bacteria from Garden Soil Sample

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Abstract

Cellulose is degraded by a group of enzymes which acts synergistically to randomly cleave the β-1, 4 glucosidic bonds. The enzyme complex responsible for the hydrolysis of cellulose is cellulases. It is an inducible enzyme secreted by wide variety of fungi, bacteria, protozoan, plants and animals. In the present study was focused on the isolation and screening of cellulolytic bacteria from soil sample and their potential for cellulase production. A totally 26 cellulolytic bacterial strains were isolated from the soil. Among them, six isolates were able to produce cellulose during SMF and they are labelled as BTSR1, BTSR2, BTSR3, BTSR4, BTSR5 and BTSR6 for further morphological and biochemical analysis. The enzyme activity was measured by the DNS method. Among the isolates, BTSR1 (1.817±0.26 U/ml) and BTSR2 (1.635±0.17 U/ml) were showed the maximum cellulose activities under SMF at 24 hours after incubation. The optimization of cellulase enzyme production of these seven isolates was carried out by using different parameters such as pH, temperature, and carbon sources. The highest enzyme activity was reported by isolates BTSR1 at pH 7 and it was found to be 3.185±0.17 U/ml. The optimum temperature for the enzyme production of isolates were BTSR1 (4.272±0.37 U/ml), BTSR2 (3.921±0.32 U/ml) and BTSR4 (3.167±0.65 U/ml) at 35 °C. CMC was used as a carbon source, maximum enzyme activity was achieved in all isolates. The highest enzyme production was noted in the isolates BTSR1 (5.431±0.97 U/ml) by using the CMC as a carbon source. The enzyme activities were also found to be almost comparable in the presence of glucose, sucrose and maltose. A maximum enzyme activity also was noted in the 24 to 36 hours incubation period. In the present study concluded that the six isolates have potential enzyme activity and also produced cellulase with different optimal conditions.

***Key words*:** CMC agar plate, Cellulase enzyme, Cellulolytic bacterial, Optimum conditions, Biochemical analysis

Lignocellulose forms the major structural component of plants. It is made up of cellulose along with hemicelluloses and lignin strongly interlocked by non-covalent and covalent forces. Annual production of lignocellulosics through photosynthesis is 40 billion tones [1]. The problem of increasing the utility of lignocellulose wastes has been known for decades. It is the most dominating waste material from agriculture and forest [2]. Lignocellulosic biomass is a major resource for the production of bio-fuels since it is largely abundant, inexpensive and eco-friendly. Lignocelluloses are employed for traditional applications (paper manufacture, biomass fuels, composting, animal feed, etc.) since long back and novel markets for lignocellulosics have been identified in recent years. In the field of biotechnology, bioconversion of cellulosic biomass to develop novel and useful products is an important area of research.

Cellulolytic organisms are generally carbohydrate degraders and are not able to use proteins or lipids as energy sources. They play a major role in biogeochemical cycling. *Cellulomonas* and *Cytophaga* can utilize a variety of other carbohydrates in addition to cellulose, but the anaerobic celluloytic species have a limited carbohydrate range, restricted to cellulose and or its hydrolysis products [3]. A promising step for the successful utilization of cellulose is the microbial hydrolysis and subsequent fermentation of the resultant sugars to produce of desirable by-products. Due to huge capital investments and extensive research, this renewable resource had proved for many commercial applications. This renewable energy has lower greenhouse gas emissions, minimize the environmental impact and can act as safe and sustainable energy.

The conversions of lignocellulosics to alternative energy resources are increasing now-a-days [4]**.** The pulp and paper industry discovered lignocellulose biotechnology could improve overall process efficiency. Research aimed at increasing digestibility of nutritionally poor forages by

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exposing these lignocellulosics to white-rot fungi [5]. Khatiwada [6] isolated cellulase producing bacterial isolates from municipal solid wastes and rice straw wastes using Carboxy Methyl Cellulose (CMC) agar medium as a selective medium. Manmeet Kaur [7] isolated cellulose degrading bacteria present from kitchen waste with highest cellulase activity. Shilpa Lokhande [8] isolated a total of 146 organisms saline soil of Akola and Buldhana District, Maharashtra. Preliminary identification was done based on Morphology and Biochemical characterization. Prem Anand [9] screened for cellulolytic and xylanolytic bacteria which help in the digestion of cellulose and xylan in Bats (*Pteropus giganteus*). The present study was focused on the screening and isolation of the potential cellulase-producing bacteria and the optimization of different parameters to maximize cellulase production.

MATERIALS AND METHODS

Chemicals

Carboxymethyl cellulose, peptone, $(NH_4)_2$ SO_4, K_2HPO_4, $MgSO_4.7H_2O$, gelatin agar, Congo sodium chloride, Tyrptone Na_2HPO_4, $CaCl_2.2H_2O$, $FeSO_4.7H_2O$, Dinitrosalicylic acid, acetate buffer, Tris-HCl, citrate buffer and phosphate buffer. All the chemicals were of analytical grade and purchased from Sisco Research Laboratories Pvt. Ltd. India.

Collection of soil sample

The soil samples were selected for isolation of cellulose producing bacteria from dump yards regions of Thanjavur, Tamil Nadu, India (Geographical coordinates are 10.726 N, 79.080 E). The samples were made at a depth within 5 cm from the surface of the soil. The collected soil samples were brought to the laboratory in sterilized polythene bags, handpicked, air dried and to pass through a sieve (2mm). They were collected in a plastic container, sealed and stored at 4 °C until further use.

Isolation of bacteria

Serial dilution technique has been used to isolate cellulolytic bacteria from soil sample about 10^{-1} to 10^{-7} dilution factor. The 50 µL of the diluted sample (10^{-5}) was plated on the screening medium composed of carboxymethyl cellulose (CMC) containing – 1.0% carboxymethyl cellulose, 1.0% peptone, 0.2% $(NH4)_2$ SO_4, 0.2% K_2HPO_4, 0.03% $MgSO_4.7H_2O$, 0.2% gelatin and 1% agar [10]. The CMC gar plates were incubated for 48 hours at 37 °C. For the primary screening of cellulose producing bacteria, point inoculation of the isolates was carried out on the same cellulose screening media plates, followed by incubation at 37°C for 48 hours. The plates were flooded with 0.1% Congo red and further washed using 1M sodium chloride to visualize the zone of clearance [11].

Identification of the bacterial isolate

The cellulase producing microorganisms were identified based on colony characteristics. Gram staining methods was performed to check the morphology of the cells and spore chain were identified by spore staining methods and also biochemically characterized by IMVIC test, catalase production test, oxidase test and Triple sugar Iron test.

Screening for cellulase enzyme production

The particular strains were further tested for their abilities to produce cellulase under submerged fermentation (SMF). The maximum cellulose production isolates were considered for the further study.

Inoculum development

The bacterial cell suspensions were harvested by aseptically adding sterile water containing 0.01% tween 80 and the final concentration of $1x10^6$ cells/ml. The maximum hydrolysis zone of these isolates is grown in 20 ml inoculum production medium at 37 °C for 24 hours. [CMC 0.5%, Tyrptone 0.20%, KH_2PO_4 0.4%, $MgSO_4.7H_2O$ 0.02%., Na_2HPO_4 0.04%, $CaCl_2.2H_2O$ 0.0001%, $FeSO_4.7H_2O$ 0.0004% and pH 7.0] [12].

Enzyme production by submerged fermentation process

CMC broth was prepared and allocates 50ml in 250 ml Erlenmeyer flasks for each of the selected isolated strains. The media were inoculated with 2.5 ml of the selected bacterial isolates from the inoculum media and incubated on 37 °C for 48 hours at 150rpm. After fermentation, the fermented broth was centrifuged to remove unwanted materials at 12000 rpm for 10 minutes at 4°C. Finally, clear supernatant was collected and used to determine enzymatic activity [13].

Cellulase activity by DNS method

The activity of cellulase was assayed using Dinitrosalicylic acid (DNS) reagent, by estimating the reducing sugars released from CMC [14]. The selected bacterial strains were inoculated in CMC broth and incubated overnight at 37 °C in a shaker. After incubation, the culture was centrifuged and the supernatant was used for cellulase assay. The 0.1ml of supernatant was mixed with 0.9ml of acetate buffer (25mM). To this, 1ml of 0.1% CMC was also added and incubated at 50 °C for 10 minutes. After incubation, the reaction was halted by adding 1 ml of DNS reagent. This was followed by the incubation of the tubes at 100 °C for 10 minutes in a water bath. The absorbance was read at 550 nm with glucose as standard. The cellulase activity was determined by using a calibration curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1µmol of glucose/minute [15].

Optimization of cellulose producing microbes under SMF

Effect of pH

The effect of pH on cellulase activity was studied by using the substrate prepared in buffer (0.05 M) of different pH ranging from 5.0 to 10.0 viz, citrate buffer (pH 5-6); phosphate buffer (pH 6-8) and Tris-HCl buffer (pH 8-10), under standard assay conditions. The residual activity (%) at each pH was calculated.

Effect of temperature

To determine the temperature optimum cellulase activity was measured at different temperatures under standard assay conditions. The temperature stability of cellulase was determined by pre -incubating an aliquot of enzyme at 20, 25, 30, 35, and 40°C for 12 to 120 h and the residual activity (%) was calculated.

Effect of carbon source

The effect of carbon source on the enzyme production was checked by preparing the production medium by replacement of the carbon source CMC, i.e., CMC with glucose, sucrose, starch, and maltose. The isolates were grown at these different production media and incubated at 37°C for 72 hours. The cell-free supernatant in each of the above case was used as crude enzyme sources to check enzyme activity.

Incubation period

The incubation period has optimized for the cellulase production and the pH of the production media broth was adjusted to 7.0. In a shaking incubator, 1 ml inoculum was then added to 50 ml of fermentation broth at 37°C with 150 rpm. The fermentation product was withdrawn at different time intervals, e.g., 16, 24, 36, 48, and 72 hours and then centrifuged at 12000 rpm for 10 minutes at 4°C. After centrifugation, for cellulase testing, the supernatant was used to approximate a standard glucose curve.

Statistical analysis

All the experiments in the present study were carried out in triplicates and the data were obtained in the form of mean ± standard error using Microsoft excel 2013.

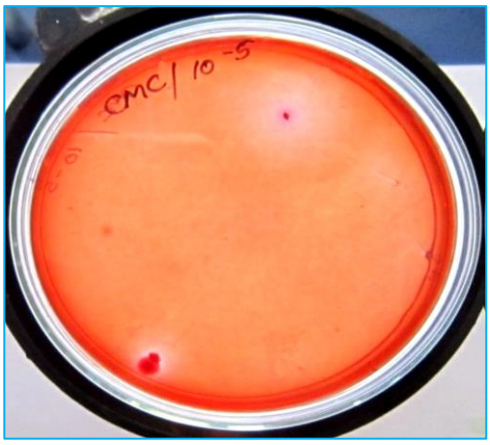

Fig 1 Hydrolysis for the substrate on the CMC – Congo red agar plate (10^{-5} dilution)

RESULTS AND DISCUSSION

Screening of cellulolytic bacteria

A total of 26 cellulolytic bacterial strains were isolated from the soil sample using the 10^{-5} dilution plate. Among them, six colonies which produced largest zone and hydrolysis the substrate CMC on CMC – Congo red agar plate by cellulolytic bacteria (Fig 1). These isolates were used for the analysis of secondary screening. Bacterial isolates showed different zones of clearance around the colonies. The zone of clearance around the colony is an indicator of cellulolytic potential of the isolates. Talia *et al.* [16] discovered that *Pseudomonas* spp. dominated in soil samples obtained from native Chaco soil. Lednická *et al*. [17] isolated and identified different cellulolytic strains from Belgian and Czech soils. The enrichment of these samples was carried out on flax or sisal fibers as sole sources of carbon.

Morphological and biochemical characterization

The isolates BTSR1 and BTSR6 were showed slow, circular shape, the surface is smooth and shiny, the margin is entire, the colour of the strain are pale yellow colour, pulvinate elevation and buttery consistency were measured. BTSR2 showed slow growth, punctiform shape, rough surface, irregular margin, white colour, umbonate elevation, adhesive consistency. BTSR4 and BTSR5 were showed the more or less both are equal characteristics. BTSR3 showed the growth rate rapid, shape, are rhizoid, smooth surface, lobate margin, milky white colour, flat elevation and viscous consistency were recorded (Table 1). BTSR1 and BTSR5 isolates were showed gram positive and BTSR2, BTSR3, BTSR4 and BTSR6 isolates observed in gram negative bacteria. Biochemical characteristics such as indole, methyl red, voges-proskauer, citrate utilization, catalase, oxidase and triple sugar iron agar test also analyzed the isolated strains (Table 2). Biochemical results observed by Arusha *et al.* [18] showed that the cellulolytic isolate was positive to indole, VP, citrate, oxidase, catalase, gelatin hydrolysis, urease and glucose utilization and negative to lactose utilization, sucrose, maltose, xylose, Arabinose, H_2S production, endospore, methyl red and Gram's reaction. Shaikh [19] observed the biochemical results of the cellulolytic strain as Gram negative short rods glucose, lactose, mannitol positive, slant and butt (TSI) and identified the organisms as *Pseudomonas*.

Table 1 Morphological characterization

Isolates	Morphological characters						
	Growth	Shape	Surface	Margin	Color	Elevation	Consistency
BTSR 1	Slow	Circular	Smooth shiny	Entire	Pale yellow	Pulvinate	Buttery
BTSR 2	Slow	Punctiform	Rough	Irregular	White	Umbonate	Adhesive
BTSR 3	Rapid	Rhizoid	Smooth	Lobate	Milky white	Flat	Viscous
BTSR 4	Rapid	Punctiform	Rough	Undulate	White	Pulvinate	Adhesive
BTSR 5	Rapid	Punctiform	Rough	Erose	White	Convex	Viscous
BTSR 6	Slow	Circular	Smooth shiny	Entire	Pale yellow	Pulvinate	Buttery

BTSR- Bio Technology Sunethra Rajakumar

Table 2 Biochemical characterization

Isolates	Gram reaction	Indole test	MR	VP	CU	Catalase	Oxidase	TSI test	Name of the species
BTSR1	+ ve	-	-	-	+	+	+	A/AH_2S	*Bacillus subtilis*
BTSR2	- ve	+	+	+	+	+	+	A/AH_2S	*Pseudomonas aeruginosa*
BTSR3	- ve	+	-	-	+	-	+	A/AH_2S	*Serratia fonticola*
BTSR4	- ve	+	+	-	-	+	-	A/A	*Escherichia coli*
BTSR5	+ ve	+	+	+	+	-	+	A/AH_2S	*Clostridium butyricum*
BTSR6	-ve	-	-	+	-	+	+	H_2S	*Staphylococcus aureus*

A/A -Acid butt Acid slant, H2S-H AL/A Hydrogen sulphide production, AL/A-Alkaline butt acid slant

Screening of cellulase production by sub merged fermentation

In this study, the possible six bacterial isolates (BTSR1, BTSR2, BTSR3, BTSR4, BTSR5 and BTSR6) were able to produce cellulose during SMF. Crude enzyme samples were tested using the enzyme activity technique. Bacterial species were collected by spreading sample dilution on CMC agar plates which it was produce the large and clear zones in shorter time were transferred in to liquid medium. The enzyme activity was measured by the DNS method. Among the strains, BTSR1 (1.817±0.26 U/ml) and BTSR2 (1.635±0.17 U/ml) were

showed the maximum cellulose activities under SMF at 24 hr after incubation. BTSR6 (1.273±0.43 U/ml) and BTSR4 (1.182±0.15 U/ml) were showed the moderate enzyme activity. Least enzyme activity was observed in the BTSR3 (0.427±0.06 U/ml) and BTSR5 (0.558±0.03 U/ml) isolates under SMF condition (Fig 2). Gupta *et al.* [20] found in their studies several cellulose-degrading bacteria with cellulase activity ranging from 0.162 to 0.400 U/ml. In contrast, another study by Ekperigin [21] demonstrated that the *A. Anitratus* and *Branhamella* spp. Produced highest enzyme activity was 0.48 and 2.56 U/ml for CMC, respectively [21]. In previous result similarly suggested that the bacterial isolates CBM21 was showed the maximum cellulase activity [13].

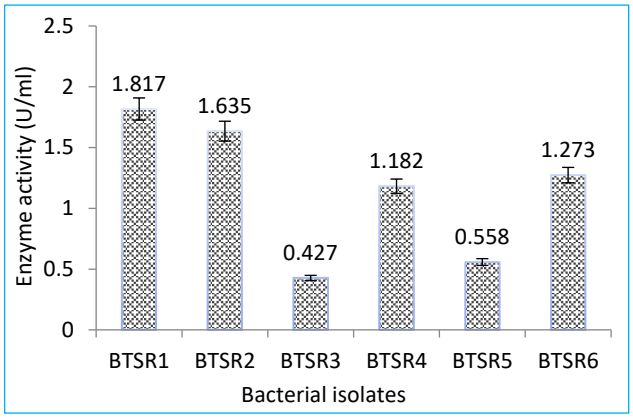

Fig 2 Enzyme activities from different isolates

Optimization of cellulase production

Effect of pH on cellulase activity

The effect of pH on cellulase production isolates were checked at different pH ranges between pH 5 to pH 10. Isolates BTSR1 (3.185±0.17 U/ml), BTSR4 (2.662±0.14 U/ml) and BTSR6 (2.817±0.16 U/ml) showed maximum production at pH 7. The highest enzyme activity was reported by isolates BTSR1 at pH 7 and it was found to be 3.185±0.17 U/ml. pH 8 was found to be optimum enzyme production by isolates BTSR2 (2.952±0.15 U/ml) and BTSR5 (2.492±0.43 U/ml). The isolates BTSR3 was found show the maximum enzyme activity at pH 6 (2.114±0.16 U/ml). The above-mentioned data suggest that slightly acidic to slightly alkaline pH, i.e., 6–8, was found to be the most suitable pH range for cellulase production (Fig 3). Shanmugapriya *et al*. [22] reported that slightly acidic to neutral pH supports maximum production of cellulase enzyme. Nandimath *et al*. [23] isolated *Bacillus* and *Pseudomonas* species having cellulolytic potential. In previous study, Abdel-Mawgoud *et al.* [24] finding that most of bacteria are best pH range for cellulase production was pH 6 to 8.

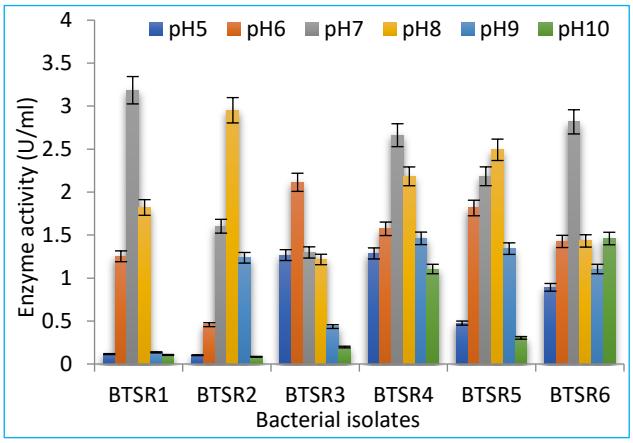

Fig 3 Effect of various pH on enzyme activity

Effect of temperature on cellulase activity

The optimum temperature for the enzyme production of isolates BTSR1 (4.272±0.37 U/ml), BTSR2 (3.921±0.32 U/ml) and BTSR4 (3.167±0.65 U/ml) at 35 °C. The highest activity at this temperature showed by isolates BTSR1. Isolates BTSR3 (2.367±0.43 U/ml) and BTSR6 (3.426±0.52 U/ml) showed the maximum enzyme production at 40 °C. BTSR5 (2.516±0.32 U/ml) showed the maximum enzyme level at 30 °C. The optimum temperature for the enzyme production of various isolates at the ranges of 35 °C to 40 °C (Fig 4). These results were found to be comparable to the findings of Premalatha *et al*. [25] wherein *Enhydrobacter* species were isolated from leaf litter compost sample for the production of cellulase enzyme and the activity was found to be highest at 30 °C. Similar results were reported by Islam *et al*. [15], in a known *Bacillus* strain where the highest cellulase production was observed at 35°C and the enzyme production was found to be reduced with an increase in the fermentation temperature above 40 °C. Rasul *et al*. [26] also reported that the enzyme activity increases gradually up to 40 °C and is found to be decreased at higher temperatures. Bakare *et al*. [27] claimed that the *Pseudomonas fluorescence* produced cellulase enzyme during 30 to 35 °C with showing the best result at 35 °C temperature. On the other hand, *Bacillus subtilis* 115 and *Bacillus subtilis* were optimally temperature at 40 °C; also, minimum cellulase yield was observed in fermentation at 45 °C [28], while *Bacillus subtilis* and *Bacillus circulans* achieved optimum yield at 40 °C [29].

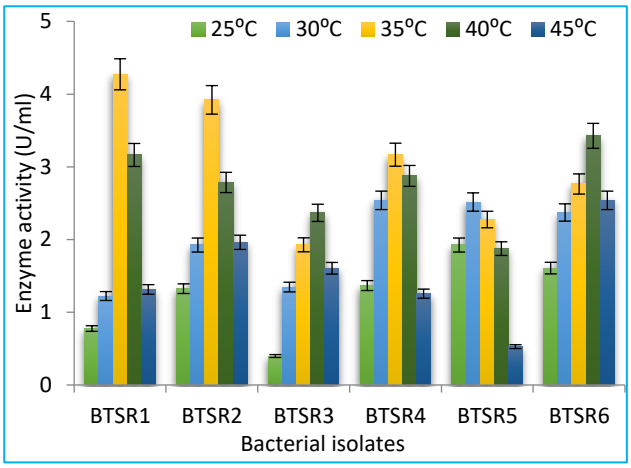

Fig 4 Effect of various temperatures on enzyme activity

Effect of carbon sources on cellulase activity

Various carbon sources such as Glucose, CMC, Starch, Sucrose and Maltose were used for the assay. Results revealed that maximum enzyme was produced and maintained when CMC was used carbon source followed by starch and glucose. When CMC was used as a carbon source, maximum enzyme activity was achieved in all isolates. The highest enzyme production was noted in the isolates BTSR1 (5.431±0.97 U/ml) by using the CMC as a carbon source. The enzyme activities were also found to be almost comparable in the presence of glucose, sucrose and maltose. Starch also produced the maximum level of enzyme production in the isolates BTSR1 (4.173±0.92 U/ml), BTSR2 (3.275±0.63 U/ml), BTSR3 (2.510±0.28 U/ml) and BTSR5 (2.972±0.76 U/ml). BTSR4 (3.315±0.42 U/ml) and BTSR6 (3.892±0.93 U/ml) showed the maximum level of enzyme production when Glucose used as a carbon sources (Fig 5). The results were comparable to Sadhu *et al*. [30]. The *Bacillus* strain under the investigation was found to produce maximum cellulase enzyme in the presence of CMC as a carbon source. Sethi *et al*. [31] found that the glucose was the best carbon source to produce cellulase enzyme by the

bacterial isolates. Shajahan *et al*. [32] reported that CMC is a crucial factor to produce cellulase enzyme. The data suggest that polysaccharides such as CMC and starch are needed for the production of cellulase enzyme.

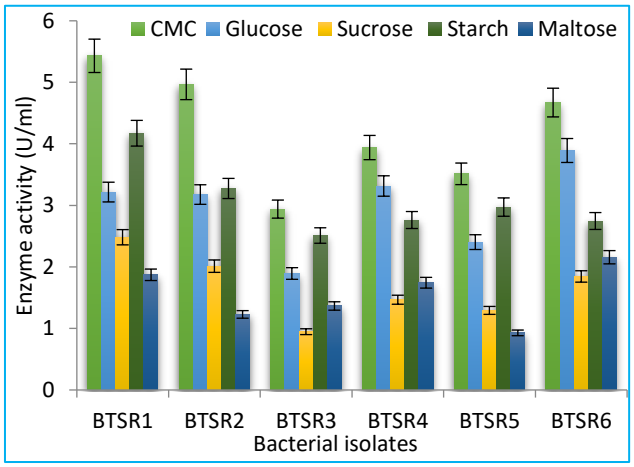

Fig 5 Effect of carbon sources on enzyme activity

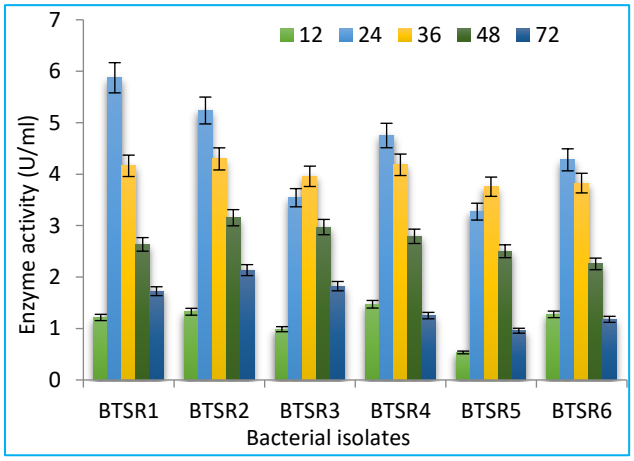

Fig 6 Effect of incubation period on enzyme activity

Effect of incubation period on cellulase activity

A maximum enzyme activity was noted in the 24 to 36 hours incubation period and after that decreased more rapidly as the fermentation its endpoint. The highest enzyme activity was noted in the BTSR1 (5.873±0.52 U/ml), BTSR2 (5.237±0.74 U/ml), BTSR4 (4.751±0.67 U/ml) and BTSR6 (4.278±0.27 U/ml) at 24 hours. The isolates BTSR3 (3.958±0.78 U/ml) and BTSR5 (3.756±0.54 U/ml) were showed the maximum enzyme level at 36 hours incubation periods. Overall, 24 to 36 hours was optimum conditions of enzyme production (Fig 6). After 48 to 72 hours decrease in enzymatic activity could be due to nutrient depletion in the fermentation medium. Haq *et al*. [34] also suggested that a decrease in incubation enzymatic activity could be due to nutrient depletion and in the fermentation medium, the formation of other by-products. At the same time, another study by Ariffin *et al.* [35] concluded that the inactivation of enzyme secretion is associated with bacterial stress caused by nutrient depletion in the medium. However, the highest activity of the enzymes was observed in our research at 24 hours of bacterial culture.

CONCLUSION

Cellulose is the major cell wall component of plant responsible for strength and rigidity. It is the predominant polysaccharide synthesized by plants. A vast group of microbes use cellulose as their source of carbon. Both aerobic and anaerobic microbes are capable of utilizing cellulose. Cellulolytic microbes are generally carbohydrate degraders and do not use proteins or lipids as energy sources. Microbial degradation of cellulosic waste is mediated by several enzymes, the most predominant of which are the cellulases. These enzymes are produced by the microbes when they are degrading the cellulosic material. In the present study, an effective cellulose degrading strains were isolated from the soil sample. The strain was able to produce appreciable amounts of cellulase when tested under laboratory conditions. The strain had wide range of optimization profiles making them promising role in future applications. The enzyme activity of the strain can be further increased by testing against more optimization profiles and gene cloning methods.

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Conflicts of interest

The authors declare no conflict of interest.

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