Advances in Microbiology, **2013, 3, 326-332**
http://dx.doi.org/10.4236/aim.2013.34046 Published Online August 2013 (http://www.scirp.org/journal/aim)

Cellulase Producing Bacteria from the Wood-Yards on Kallai River Bank

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Received May 25, 2013; revised June 25, 2013; accepted July 10 2013

ABSTRACT

This study evaluates the influence of growth parameters such as pH, temperature, Carboxy Methyl Cellulose (CMC) concentration and agitation on cellulase production from three bacterial strains, *viz*., *Achromobacter xylosoxidans* BSS4, *Bacillus* sp. BSS3 and *Pseudomonas* sp. BSS2 isolated from the wood-yards on Kallai river bank in Kerala. Production of cellulase by these isolates was detected using basal salt medium (BSM) with 0.5% CMC as supplement, and CMCase activity was confirmed by iodine test. Dinitrosalicylic acid method was employed for assaying the cellulase production by measuring the amount of glucose liberated in µmol/mL/min. Maximum enzyme production from *Pseudomonas* sp. BSS2 was at pH 8, 37˚C with 1% CMC and 150 rpm, and cellulase production increased from initial 49.84 U/mL to 91.28 U/mL after optimization. The highest enzyme activity from *Bacillus* sp. BSS3 was at pH 9, 37˚C with 1% CMC, 150 rpm, and cellulase production increased from initial 26.05 U/mL to 104.68 U/mL after optimization. The maximum enzyme production from *A. xylosoxidans* BSS4 was at pH 7, 40˚C with 0.5% CMC and 150 rpm, and cellulase production increased from initial 55.28 U/mL to 68.37 U/mL after optimization. Thus among the three isolates, *Bacillus* sp. BSS3 showed maximum enzyme yield which can be explored for further scale up studies with an industrial perspective.

Keywords: Cellulase; Carboxy Methyl Cellulose; Dinitrosalicylic Acid; Optimization; Submerged Fermentation

1. Introduction

Lignocellulose, the leading bio-residue from agricultural sector is the predominant renewable biopolymer in the world which comprises of celluloses, hemicelluloses and lignin. A promising strategy for the efficient utilization of this renewable resource is to use it as a base material for the production of desired metabolites. Apart from the production of value-added products, its bioconversion offers an effective solution for the abatement of pollution due to solid-waste and their utilization, which would allow sustainable process and products. Numerous products of high economic value like alcohols, acids, single cell proteins, paper, etc., are produced by the effective bioconversion of lignocellulosics [1]. Cellulose is the primary product of photosynthesis in plants which is a polymer having D-anhydroglucopyranose molecules linked by β-1,4-glycosidic bonds [2]. Eventhough bulk quantity of cellulosic residues gets accumulated in the terrestrial ecosystem, they are actively degraded by numerous bacteria and fungi, then contributing to maintaining the carbon cycle.

Cellulose is degraded by an enzyme system called cellulases produced by fungi, bacteria and actinomycetes. The cellulase system constitute three major enzymes; *i.e*., endoglucanase (endo-1,4-β-D-glucanase, EC3.2.1.4), exoglucanase (exo-1,4-β-D-glucancellobiohydrolase, EC3.2.1.91), and β-glucosidase (β-D-glucoside glucanohydrolase, EC 3.2.1.21), which act synergistically towards the complete breakdown of cellulose. Endo-glucanases make nicks within the cellulose biomolecule thereby exposing their reducing and non-reducing ends, cellobiohydrolases release cellobiose units—a disaccharide of two glucose molecules linked by a β-1,4 linkage—the repeating units of cellulose from the chain ends; and finally β-glucosidases act on cellobiose to liberate glucose [3].

A wide variety of microorganisms have the ability to degrade cellulose, which include aerobic and anaerobic bacteria, white-rot and soft-rot fungi. Fungi are the most studied organisms with respect to the production of cellulolytic enzymes. Compared to fungi, bacteria have numerous advantages on an industrial view point like its

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high growth rate, easy handling and adaptability to various genetic manipulations [4]. Production of extracellular enzymes, especially carboxymethyl cellulase (CMCase) by aerobic bacteria like Bacillus and Cellulomonas [5], are advantageous for large-scale applications. Bacterial cellulases are constitutively produced by submerged fermentation (SmF) in industries employing mainly genetically modified strains. Most importantly, thermophilic, psychrophilic, alkalophilic, acidophilic and halophilic bacteria inhabit a wide variety of environmental and industrial niches that are extremely resistant to environmental stress and they can produce enzymes which are stable under extremely harsh conditions [6]. As a result, isolation and characterization of cellulase-producing bacteria will continue to be a principal component of enzyme research.

Compared to solid-state fermentation (SSF), SmF is widely used in industries since it is easy to operate with control over various process parameters, coupled with easy downstream processing [7]. Various process parameters like incubation time, temperature, pH, agitation, etc., seem to influence microbial growth and production of cellulase; thus a judicious selection of these parameters can dramatically improve the enzyme yield.

The centuries-old wood-yards on the Kallai river banks, in the suburbs of Kozhikode City, Kerala State, India, are famous for timber-based industries and allied business. Pursuant to that, enormous quantity of lignocellulolytic wastes are being created day-by-day in the form of carpentry waste, sawdust, wood chips etc. Being marshy and almost anoxic environment with wide variation in pH, it is expected that extremophiles could be isolated and characterized from this habitat, and that by centuries-old natural processes, enormous microbial wealth (bacteria, fungi and yeast) thriving on wood would have evolved in this environment, and thus the importance of this study. In the light of this background, the present study focuses on the isolation and characterization of novel bacteria from this wood-yard with potentials for producing cellulase.

2. Materials and Methods

2.1. Sample Collection

Samples rich in cellulose content like wood bark of timber on river bank side, water-logged wood, sawdust, and sludge from sawdust dumping site were collected from five different locations near the milling areas of wood-yards on Kallai river side.

2.2. Isolation

Samples were transported to the lab under sterile conditions and one gram of the sample was transferred in a 250 mL flask containing sterile double distilled water (ddH_2O), which was made up to 100 mL. The mixture in the flask was shaken for 15 - 20 min (150 rpm at 37˚C) for getting detached the attached surface microflora. One mL of this sample was plated after sufficient serial dilutions (up to 10^{-7}) on Mullen Hinton Agar (MHA) plates and incubated for 24 - 48 h at 37˚C.

2.3. Screening for Cellulolytic Activity

Bacterial cultures grown on MHA slant were cultured on basal mineral salt medium (BSM) containing (g/L) 2.0 $NaNO_3$; 1.0 K_2HPO_4; 0.5 KCl; 0.5 $MgSO_4 \cdot 7H_2O$; 2.0 proteose peptone; 20 agar and 0.5% CMC as additional nutrient, *i.e.*, for the microbial screening for cellulase activity. Detection of CMCase activity was performed on the culture plate using iodine test (solution containing 1% iodine crystals and 2% potassium iodide), which would in 15 min form a bluish-black complex with unused CMC, demarcating a clear zone around the colonies [8,9].

2.4. Characterisation of Bacteria

Characterization of bacterial isolates was based on cell morphology and biochemical tests. Gram staining, production of endospore, motility, IMViC, catalase, production of H_2S, carbohydrate fermentation (glucose, lactose, sucrose and mannose [G, L, S, M]) starch and casein hydrolyses were the tests employed for the characterization of bacteria.

2.5. Molecular Characterization

The isolates were confirmed by the PCR-amplification of 16S rDNA gene from the isolated genomic DNA with 8F, and 1492R primers using BDT v3.1 cycle sequence kit on ABI 3730 × 1 genetic analyser (Xcelris Labs, Ahmedabad, India).

2.6. Optimization of Cellulase Production

Different parameters applied for SmF were optimized for enhancing cellulase production.

2.6.1. Effect of pH

To determine the optimum pH for cellulase production, BSM containing 0.5% CMC with different pH, *i.e.*, 4, 5, 6, 7, 8, 9 or 10 was inoculated and incubated in the shaker at 37˚C. Whole flask samples were withdrawn and cellulase activity in the supernatant was assayed at every 6 h interval.

2.6.2. Effect of Temperature

To determine the optimum temperature for cellulase production, BSM containing 0.5% CMC was prepared with

optimum pH fixed earlier for each culture was incubated at 35˚C, 37˚C or 40˚C in a shaker. Whole-flask samples were withdrawn at every 6 hours interval for the cellulase assay.

2.6.3. Effect of CMC Concentration

To determine the optimum CMC concentration for cellulase production, BSM with different concentrations of CMC (0.5%, 1% or 1.5%) was prepared and inoculated with bacterial cultures and incubated at pH and temperature fixed earlier for each culture, and whole flask samples were withdrawn at every 6 h interval for the cellulase assay.

2.6.4. Effect of Agitation

To determine the effect of agitation in the production of cellulase, BSM having CMC was prepared with optimum pH, temperature and CMC concentration fixed for each culture and kept along with appropriate controls with agitations (50, 100, 150 or 200 rpm).

2.7. Enzyme Assay (CMC as Substrate) by DNS Method

Cellulase activity was determined by extracting the supernatant by centrifugation at 8944 g at 4˚C. The reaction mixture contained 0.5 mL of 1% CMC (1 g CMC in 100 mL of 0.1 M citrate buffer, pH 4.8) as substrate; 0.5 mL of crude enzyme (supernatant) was added to it and incubated at 50˚C for 30 min in a water bath. At the end of the incubation period, 3 mL of 3,5-dinitrosalicylic acid (DNS) was added and incubated for 5 min in a boiling waterbath for color development and cooled rapidly. The reducing sugar was measured by the method of Miller [10]. The activity was measured against a reagent blank at 540 nm in a UV-Vis spectrophotometer (Shimadzu, Japan). One unit of cellulase activity is defined as the quantity of cellulase required to liberate 1 µmol of glucose equivalents per minute under the assay conditions. Cellulase activity was calculated using the formula, $\Delta E \times V_f/\Delta t \times \sum \times V_s \times d$; where, ΔE = absorbance at 540 nm, V_f = final volume of reaction mixture, including DNS, Vs = crude supernatant (mL) containing cellulase used, Δt = incubation time for hydrolysis, $\sum$ = extinction coefficient of glucose (0.0026), d = diameter of cuvette.

2.8. Statistics

All experiments were repeated 5 times, and average values with SD were plotted on the graphs.

3. Results

In the present study, we isolated more than hundred bacteria from the wood-yards on Kallai river bank. Among them, three potent cellulolytic bacterial cultures were screened out for further studies. Characterization of the cultures was done at morphological, biochemical and molecular levels. Based on molecular characterization, the cultures were identified and named as *Achromobacter xylosoxidans* BSS4 (GenBank Accession No. JQ 407052), *Bacillus* sp. BSS3 (GenBank Accession No. JQ 407051) and *Pseudomonas* sp. BSS2 (GenBank Accession No. JQ 407050). *Pseudomonas* sp. BSS2 was obtained from the wood bark of water-logged wood, while *Bacillus* sp. BSS3 and *A. xylosoxidans* BSS4 were isolated from the bark of the woods kept partially immersed in the river bank. No potent cellulolytic isolates were obtained from other samples like sawdust and sludge collected.

The bacterial cultures were also characterized based on colony nature, shape of colony margine, size, transparency, color, shape and elevation (**Table 1**). The cultures were stored initially on MHA and later they were maintained in MSM supplied with CMC to induce cellulase production. The morphological characterization studies included Gram's reaction, and examining cell shape, endospore formation, spore position and motility. *Pseudomonas* sp. BSS2 is Gram −ve, motile rod without endospores; *Bacillus* sp. BSS3 is Gram +ve, motile rod with endospores, while *A. xylosoxidans* BSS4 is Gram −ve, motile rod with endospores (**Figure 1**). The biochemical characterization of all the three cultures showed similar response for catalase, H_2S production, hydrolysis of starch and casein, while showed variations in IMViC and carbohydrate fermentation reactions (**Table 2**). The iodine test (plate assay) for screening of cellulase activity was performed on BSM with CMC agar, in which all the three cultures showed well-defined clear zone around the colony, which indicates that the CMC in the clear zone area was hydrolyzed by the cellulase produced by the bacteria. The unutilized CMC around the clear zone formed dark colored complex with iodine. Among them, *Pseudomonas* sp. BSS2 showed wider hydrolytic zone (32 mm) (**Figure 2**).

The optimization of process parameters for cellulase production by each culture included effects of pH, temperature, CMC concentration and agitation. Our strategy was to fix one parameter first (with other parameters arbitrary), and use this fixed parameter for fixing the second one and so on. Initially, effect of pH on the enzyme

Table 1. Culture characteristics of bacterial isolates on MHA plates.

Culture name	Characteristics
Pseudomonas sp. BSS2	Creamy, opaque, mucoidal colonies, circular and convex
Bacillus sp. BSS3	Widely spreading, opaque, mucoidal colonies with entire margin
Achromobacter xylosoxidans BSS4	Creamy, opaque, circular shaped colonies with convex elevation

Table 2. Biochemical characteristics of bacterial isolates.

Culture name	IMViC	Catalase	H_2S production	Starch hydrolysis	Carbohydrate fermentation [G, L, S, M]	Casein hydrolysis
Pseudomonas sp BSS2	−, +, −, +	+	−	−	+, +, −, +	−
Bacillus sp BSS3	−, +, −, −	+	−	−	+, −, −, −	−
Achromobacter xylosoxidans BSS4	−, +, −, −	+	−	−	+, −, −, +	−

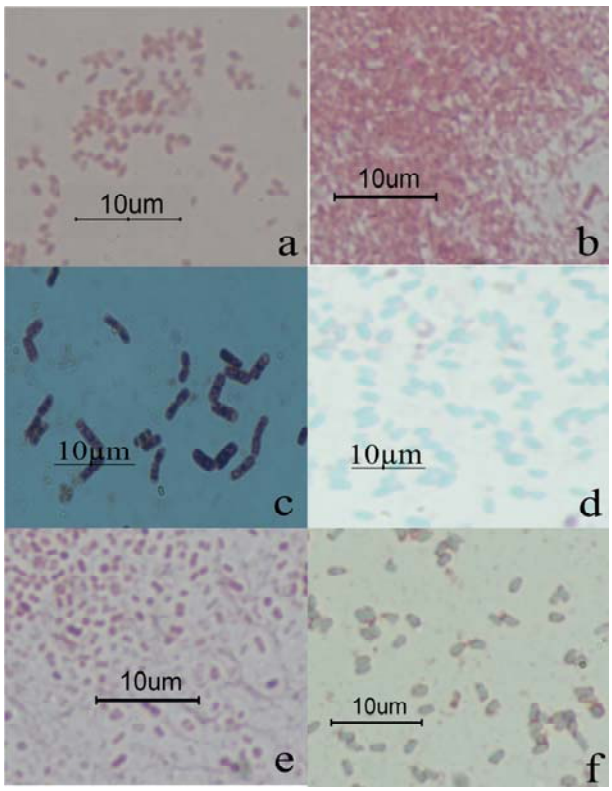

Figure 1. Morphological characteristics of *Pseudomonas* sp. BSS2, *Bacillus* sp. BSS3 and *A. xylosoxidans* BSS4. (a) Gram's staining for *Pseudomonas* sp. BSS2; (b) Endospore staining for *Pseudomonas* sp. BSS2; (c) Gram's staining for *Bacillus* sp. BSS3; (d) Endospore staining for *Bacillus* sp. BSS3; (e) Gram's staining for *A. xylosoxidans* BSS4; and (f) Endospore staining for *A. xylosoxidans* BSS4. *Pseudomonas* sp. BSS2 was gram negative and did not produce endospore, while *Bacillus* sp. BSS3 and *A. xylosoxidans* BSS4 were Gram positive with endospores.

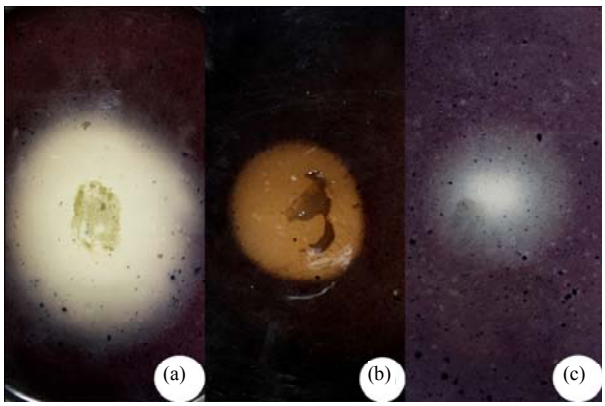

Figure 2. Iodine test for detecting cellulase activity of bacterial cultures. (a) *Pseudomonas* sp. BSS2; (b) *Bacillus* sp. BSS3; and (c) *A. xylosoxidans* BSS4 with cellulolytic activity was selected using iodine test, which formed a bluish-black complex with unused cellulose (CMC in the medium) leaving a sharp and distinct zone around cellulase producing microbial colonies on BSM-agar plate with 0.5% CMC. Clear zone mean no CMC, *i.e.*, utilized by cellulase produced by the culture.

production was analyzed from pH 4 to 9 for *Pseudomonas* sp. BSS2 and *A. xylosoxidans* BSS4; while for *Bacillus* sp. BSS3, it was analysed from pH 4 to 10, since the culture showed maximum production at pH 9. *Pseudomonas* sp. BSS2 showed maximum cellulase activity (49.85 U/mL) at pH 8 and 12 h incubation, while *A. xylosoxidans* BSS4 showed maximum cellulase activity (55.282 U/mL) at pH 7 and 6 h of incubation. *Bacillus* sp. BSS3 showed maximum activity (26.05 U/mL) at pH 9 and 6 h incubation (**Figure 3(a)**).

Effect of temperature on the enzyme production was analyzed at varying temperatures (30˚C, 37˚C and 40˚C); *i.e.*, after fixing the optimum pH for three cultures. All cultures showed maximum enzyme yield at 37˚C at their respective optimum pH fixed. *Pseudomonas* sp. BSS2 showed maximum production (59.07 U/mL) after 12 h incubation, whereas *Bacillus* sp. BSS3 (34.56 U/mL) and *A. xylosoxidans* BSS4 (61.94 U/mL) showed maximum cellulase production after 6 h incubation (**Figure 3(b)**). Effect of different CMC concentrations on cellulase production was evaluated, after fixing the optimum pH and temperature for each culture. *Pseudomonas sp.* BSS2 (62.35 U/mL) and *Bacillus* sp. BSS3 (90.10 U/mL) showed maximum activity with 1% CMC at 12 h and 6 h, while *A. xylosoxidans* BSS4 showed optimum CMC concentration as 0.5% (63.89 U/mL) at 6 h (**Figure 3(c)**). For studying the effect of agitation, the cultures were incubated at different rpm (50, 100, 150 and 200). All the three cultures showed maximum enzyme production at 150 rpm in combination with already fixed respective pH, temperature and CMC concentrations. At this standardized conditions, *Pseudomonas* sp. BSS2 showed maximum cellulase activity (91.28 U/mL at 12 h), whereas *Bacillus* sp. BSS3 and *A. xylosoxidans* BSS4 showed maximum cellulase activity at 6 h; *i.e.*, 104.68 U/mL and

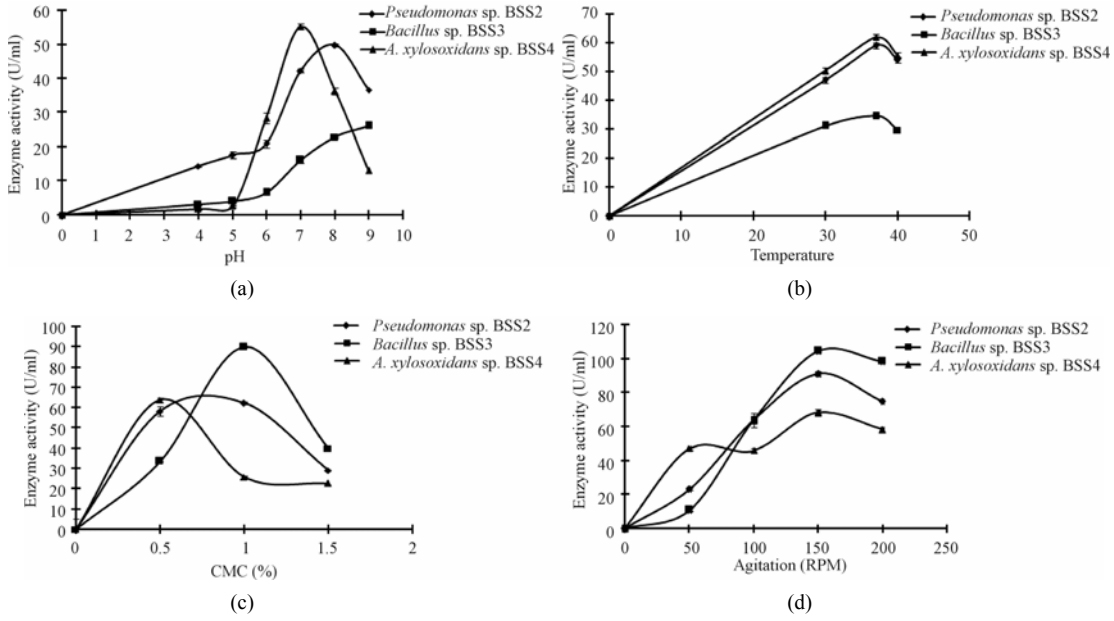

Figure 3. Optimization of parameters for cellulase production. (a) Effect of pH on cellulase production from *Pseudomonas* sp. BSS2, *Bacillus* sp. BSS3 and *A. xylosoxidans* BSS4 in BSM supplemented with CMC as substrate at pH range 4 - 10. *Pseudomonas* sp. BSS2 showed maximum cellulase production at pH 8 on 12th h of incubation, whereas *Bacillus* sp. BSS3 and *A. xylosoxidans* BSS4 showed at pH 9 and 7 on 6th h of incubation, respectively. (b). Effect of temperature on cellulase production from *Pseudomonas* sp. BSS2, *Bacillus* sp. BSS3 and *A. xylosoxidans* BSS4 in BSM supplemented with CMC. *Pseudomonas* sp. BSS2 showed maximum cellulase production at pH 8 on 12th h of incubation at 37˚C, whereas *Bacillus* sp. BSS3 and *A. xylosoxidans* BSS4 showed maximum production at pH 9 and 7, respectively (6th h of incubation at 37˚C). (c) Effect of substrate concentration on cellulase production from *Pseudomonas* sp. BSS2, *Bacillus* sp. BSS3 and *A. xylosoxidans* BSS4 in BSM supplemented with 0.5%, 1% and 1.5% CMC. *Pseudomonas* sp. BSS2 and *Bacillus* sp. BSS3 showed maximum cellulase production with 1% CMC on 12th h and 6th h at pH 8 and 9, respectively at 37˚C, whereas *A. xylosoxidans* BSS4 showed highest production with 0.5% CMC on 6th h at pH 7 and 37˚C. (d) Effect of agitation on cellulase production from *Pseudomonas* sp. BSS2, *Bacillus* sp. BSS3 and *A. xylosoxidans* BSS4 in BSM supplemented with CMC at different rpm 50, 100, 150 and 200. *Pseudomonas* sp. BSS2, *Bacillus* sp. BSS3 and *A. xylosoxidans* BSS4 showed maximum cellulase production with optimum pH, temperature and CMC concentration on 12th h and 6th h, respectively at 150 rpm.

68.22 U/mL, respectively (**Figure 3(d)**). There was no considerable difference in cellulase production from *Bacillus* sp. BSS3 and *A. xylosoxidans* BSS4 at 6 and 12 h, while it decreased thereafter upon further incubation.

4. Discussion

In recent years, more attention has been directed toward screening of novel microbial strains that have broad spectrum of enzyme activities. The high cost of cellulase production and low enzyme activities limit their Industrial use. So efforts are to be taken to economize and increase the yield of cellulase production by media optimization [11], and hence isolation, characterization and media optimization for cellulase producing bacteria remain to be an important area of biofuel research [12].

In this study, we focussed on the isolation of novel cellulase producing bacteria with an industrial perspective. So we have selected wood-yards on Kallai river—famous for numerous timber based industries—as our site for sample collection. There are reports on the isolation of cellulase producing bacteria from a wide variety of sources like compost, decayed plant material, soil, etc. [13]. Bacteria present an attractive potential for the exploitation of cellulases due to their rapid growth. The pure cultures obtained were characterized at morphological, biochemical and at molecular levels. We wanted to explore whether these cultures embodied with potentials for cellulolytic activities for which we adopted plate assay [14]. Exoglucanases are potent cellulases which are active on amorphous regions of cellulose and their activity can be assayed using soluble cellulose substrates like CMC. Screening for bacterial cellulase activity by microbial isolates is typically performed on CMC containing culture plates [8]. In accordance with this, we used basal mineral salt medium (BSM) supplemented with CMC as sole carbon source for screening of cellulolytic activity of these isolates. Hydrolytic zone on culture plates serves as a strong evidence of the hydrolytic capablities of the isolates [9].

SmF is the conventional fermentation strategy in the presence of excess water, and in comparison to SSF, it is better to monitor and handle the culture, which makes it

suitable for large scale industrial production of microbial enzymes. SmF helps in the production of cellulase and other enzymes [15]. So we selected SmF for the production of cellulase from these isolates. Cellulase production is highly influenced by various process parameters like pH, temperature, substrate concentration, agitation, *etc*.; and large scale production requires understanding and proper controlling of growth parameters to increase the enzyme production [16]. Cellulase production appears to depend on pH value. Results illustrated in **Figure 3** clearly show that cellulase production gradually increased as the pH values increased from 4 to 8 and dropped at pH 9 for *Pseudomonas* sp. and *A. xylosoxidans*, whereas *Bacillus* sp. showed maximum production at pH 9. Yang, *et al*. [17] reported that cellulase production was high between 7 and 9 for *Bacillus* sp. with a yield of 49 U/mL at pH 9. Acharya and Chaudhary [18] studied on the cellulase activity of two novel strains isolated from hot springs, *i.e.*, *B. licheniformis* WBS1 and *Bacillus* WBS3, which showed maximum cellulase activity of 0.388 and 0.342 IU/mL at pH 8 and 9, respectively. Like pH, temperature is also an important factor which influences cellulase yield.

It is obvious from **Figure 3** that cellulase production increased with increasing temperature from 30˚C to 37˚C, but decreased at 40˚C. This was in contrast to the reports of Immanuel [19], and they recorded maximum endoglucanase activity for *Cellulomonas*, *Bacillus* and *Micrococcus* at 40˚C and at neutral pH with an enzyme yield of 0.0336, 0.0196 and 0.0152 U/mL, respectively. Fagade and Bamigboye [20] observed highest cellulase activity for *B. licheniforms* I and II at 40˚C with a value of 0.52 mg/mL and 0.44 mg/mL reducing sugar. We tried CMC as supplement at different concentrations, *i.e*., 0.5%, 1% and 1.5%. For *Pseudomonas* sp. and *Bacillus* sp. optimum CMC concentration was 1%, while *A. xylosoxidans* showed maximum cellulase activity at 0.5% CMC concentration. Lin, *et al.* [21] reported that *B. thuringiensis* produced maximum relative cellulase activity of 110 U/mL at 1% CMC and 40˚C.

Agitation increases aeration in the medium, and thus helped in improving contact between substrate and microorganism, which ultimately favours better enzyme yield. In accordance with that, we obtained better enzyme yield as agitation (rpm) was increased gradually from 50 to 150 though 100 rpm, while at 200 rpm, the enzyme yield was decreased. Hence, 150 rpm was found suitable for the isolates described herein for better production of cellulase. Taleb, *et al.* [22] reported that strains of *B. alcalophilus* and *B. amyloliquefaciens* showed maximum cellulase activity (2.32 and 2.97 IU/mL, respectively) at 1% CMC and 150 rpm. Ray, *et al.* [23] reported that cellulase yield was higher in *B. subtilis* (26 U) and *B. circulans* (20.2 U) upon SSF at an optimum pH range of 7.0 to 7.5 and 40˚C temperature. Shankar, *et al.* [24] reported that *B. pumilus* EWBCM1 isolated from the gut of earthworm showed maximum cellulase activity (0.585 IU/mL) at pH 6 and 37˚C. Satheesh, *et al.* [25] studied the cellulase production by a newly isolated strain of *Bacillus* sp. using cellulose powder, rice husk and filter paper as substrates and found that rice husk was the most suitable substrate for cellulase production. Otajevwo, *et al.* [26] reported that isolates such as *Bacillus*, *Clostridium*, *Pseudomonas* and *Erwinia* showed optimum cellulase production at 40˚C and pH 6.

In conclusion, we obtained three strains, *viz*., *Pseudomonas* sp., *Bacillus* sp. and *A. xylosoxidans* with cellulolytic activity, which more promising than the reported cultures of related genera. Of these, *Bacillus* sp. showed reasonably high cellulolytic activity. Several microorganisms capable of converting cellulose into simple carbohydrates had been discovered for decades. However, demands from the enzyme industry for newly isolated cellulolytic microbes which can better convert cellulose in to value added products still active and there lies the importance of this study.

5. Acknowledgements

The authors are thankful to the Kerala State Council for Science, Technology and Environment for a research grant, No. (T) 422/SRS/2009/CSTE.

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