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J. Sci. Trans. Environ. Technov. 2015, 9(1) : 5-12

Scientific Transactions in Environment and Technovation

Cellulase production by a manglicolous actinobacterium: Optimization, characterization and partial purification

https://doi.org/10.20894/STET.116.009.001.002
www.stetjournals.com

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Abstract

Nine actinobacterial strains were isolated from the mangrove sediments of Bhitarkanika, Odisha. One of them was most potent with cellulolytic activity and it was identified as a species close to *Streptomyces minoensis* (BKM9), based on its colony morphology, biochemical and physiological characteristics. The strain was grown under different carbon, nitrogen and amino acid sources, pH and temperature to find out the optimum conditions for cellulase production. It was evident from the study, that maltose (485IU/l), yeast extract (595IU/l) and phenyl alanine (19.2 IU/ml) could be used as major carbon, nitrogen and amino acid sources respectively in fermentation at pH 7 and an incubation temperature of 37°C.

Key words : Mangroves, *Streptomyces*, cellulase, cellulolytic activity.

INTRODUCTION

Microbial enzymes have attracted the attention of the researchers all over the world because of their wide range of physiological, analytical and industrial applications, facilitated by their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation (Eichler, 2001). Marine microbes are capable of catalyzing various biochemical reactions with novel enzymes such as amylase, deoxyribonuclease, lipase, cellulase and protease (Chandramohan, 1997). Of the many microbes, actinobacteria possess a rich source of antibiotics, enzymes and bioactive molecules which are of considerable industrial importance (Nolan and Cross, 1988). So, marine actinobacteria have become important in the search of novel microbial products and hence both academic and application interests, in studying the marine microorganisms, are on the rise.

With the advent of new frontiers in the field of biotechnology, spectrum of cellulase use has expanded in various industries including food, textiles, laundry, pulp, paper, agriculture as well as research and development (Bajpai, *1999;* Bhat, *2000*). Cellulases are produced by an array of microbes, and aerobic fungs *Trichoderma* sp. (Bauchop and Mountfort, 1981) and thermotolerent *Streptomyces* sp. Ab106 (Techapun *et al.,* 2002) were found to be good producers of cellulase.

So, there is an imperative need for the exploration of microorganisms from pristine environments, for obtaining commercial enzymes (Leghlimi, 2013), including coastal and marine realms. In the present study, we report about an actinobacterial strain, associated with the mangrove sediments, as a potential cellulase producer.

MATERIALS AND METHODS

Isolation of actinobacteria

Sediment samples were collected from Dangumal (Lat. 20° 43′ 55.4" N and Long. 86°52′ 17.3" E) of the Bhitarkanika mangroves, Odisha; air-dried aseptically for one week and incubated at 55^{0}C for 5 min (Balagurunathan, 1992). The pre-treated samples (1g) were serially diluted (Jensen *et al.*, 1991) and spread on Actinomycetes Isolation agar (AIA, Hi-Media, Mumbai) and Kuster's agar (Kuster and Williams, 1964) by spread plate method. To minimize the bacterial and fungal contaminations, all the agar plates were supplemented with 20 mg l^{-1} and 100 mg l^{-1} of nystatin and cycloheximide respectively (Kathiresan *et al.,* 2005).

Identification of cellulase producing actinobacterium

Genus level identification of the potential actinobacterial strain was made using chemotaxanomy and micromorphological studies (Lechevalier and Lechevalier, 1970). Characterization of the cellulase producing actinobacterium was made following the method described by Shirling and Gottlieb (1996). Species level identification was done based on the keys of Nonomura (1974).

Screening for cellulase producing actinobacteria

Cellulase activity of the strains was screened qualitatively in CMC (Carboxy Methyl Cellulose) agar medium. After inoculation, the plates were incubated at 37^{o}C for 5 days. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1 M NaCl (Apun *et al.,* 2000). To indicate the cellulase activity of the strains, diameter of the clear zones formed around the colonies on CMC agar was measured.

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Enzyme assay

Isolates showing appreciable cellulase activity, as evidenced by the diameter of the zone of clearance greater than 30 mm, were selected for the estimation of the cellulase activity using Carboxy Methyl Cellulose (CMC) broth medium (Per liter: CMC: 10 g; KH_2PO4: 1 g; NaCl: 2 g; $MgSO_4$.H2O: 1 g; $MnSO_4$: 0.05 g; $FeSO_4$.7H2O: 0.05 g $CaCl_2$.2H2O: 2 g; NH_4Cl: 2 g; pH 7-7.4). The medium was inoculated with 1 ml of spore suspension (10^7spores/ml) of a 7 days old culture and incubated under shake culture condition on a rotary shaker with a speed of 150 rpm, at the ambient temperature of 37°C, for 72 h.

Cell Free Supernatants (CFS) obtained after centrifugation at 12,000g for 15 min, using a refrigerated centrifuge, were used for the estimation of cellulase activity with the substrate carboxymethylcellulose (CMC).

Cellulase activity was assayed by measuring the release of reducing sugars in a reaction mixture of 1.0 ml of the crude enzyme and 1.0 ml of 2% (w/v) CMC solution in 50 mM phosphate buffer (pH 7), incubated at 37°C, for a period of 10 min. The amount of reducing sugar produced was measured by the 3, 5-dinitrosalicylic (DNS) reagent method (Miller, 1959). One international unit (IU) of enzyme activity for cellulase was defined as the amount of enzyme releasing 1 µm reducing sugar from CMC per minute using glucose as standard. Protein concentration was determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Purification of cellulase

The potential strain was grown in the production medium for 72 h at 37°C in a rotary shaker. After incubation, CFS was obtained as described earlier. The enzyme from CFS was precipitated by ammonium sulfate at 60–80% saturation at 4°C. The precipitate was collected by centrifugation at 12,000×g for 15 min at 4°C and dissolved in 1 mM Tris buffer (pH 7.0). This aliquot was dialyzed against the same buffer overnight under refrigerated conditions. Partially purified enzyme was applied to a Sephadex G-100 column equilibrated with 1 mM Tris buffer (pH 7.0). Equilibration and elution were performed with 1 M Tris buffer (pH 7.0) containing 0.1 M NaCl. Elution was carried out using the same buffer at a flow rate of 0.5 ml/min. All the purification procedures were carried out at 4°C. Finally, the resulting enzyme preparation was desalted, concentrated by dialysis and lyophilized.

Determination of molecular weight of the purified enzyme

Molecular weight of the partially purified enzyme was determined by denaturing (SDS-PAGE), (Laemmli, 1970). Molecular weight was determined by comparing the relative mobility of the standard protein of the low molecular weight markers (Bio-Rad, 1993).

Optimization of growth parameters for cellulase production

Fermentation experiment was carried out with the important parameters up to 10 days and the production rate was measured at 24 h intervals, for the optimization of the growth parameters.

Temperature

In order to determine the effective temperature for cellulase production by the actinobacterial strain, fermentation was carried out at 25, 28, 30, 35, 40, 45 and 50 °C for 7 days.

Carbon and nitrogen sources and amino acids

Production of cellulase, under different carbon and nitrogen sources availability, was studied with 1% carbon sources (galactose, glucose, fructose, glycerol, sucrose and maltose), 1% nitrogen sources (peptone, yeast extract, and beef extract) and 1% amino acids (L-glutamine, L-asparagine, L-phenylalanine, L-tyrosine and L-histidine). After incubation at appropriate conditions, the cells were removed by cold centrifugation at 12,000g for 15 min. Resultant supernatant was analysed for cellulase activity.

RESULTS AND DISCUSSION

Isolation of cellulase producing actinobaterium

Biotechnological applications of cellulases began in the early 1980s in animal feed followed by food applications (Harchand and Singh, 1997). Today, these enzymes account for approximately 20% of the world's enzyme market. In line with this, present study was carried out to isolate potential actinobacterial strains from the Bhitarkanika mangrove sediments, Odhisa, India. Earlier, Vijayakumar *et al.* (2007) isolated 192 actinobacterial strains from the mangrove sediment samples of the Palk Strait, Bay of Begal. From the Muthupet mangroves of the Palk Strait region, situated along the South East coast of India, 57 actinobacterial isolates were obtained (Priya *et al.,* 2014). From the Konkan coast of Maharashtra, Gulve and Deshmukh (2011) reported 90 isolates belonging to different genera *viz. Streptomyces, Micromonospora, Intrasporangium, Sachharopolyspora, Streptosporangium, Rhodococcus, Sachharomonospora* and *Nocardia* which possessed protease (84.44%), gelatinase (77.78%), amylase (72.22%), lecithinase (43.33%), cellulase (37.78%) and ureas (16.67%) activity.

Mohanta (2014) has reported nine isolates of cellulose degrading actinobacteria from the Bhitarkanika mangrove area and fifty five actinobacterial strains have been isolated from the Nizampatnam and Coringa

mangroves, south east coast of Andhra Pradesh (Sharma, 2014). Recently, 43 actinobacterial strains were recorded from the mangrove sediment samples of Bhiterkanika. Among them, 19 isolates were tested for amylase, cellulase, protease, lipase, L-asparginase, L-glutaminase, chitinase and pectinase activity (Saranya *et al*., 2015). These studies reveal that the mangrove sediments which contain rich sources of nutrient and organic matter might provide with suitable niches for the actinobacterial growth.

Identification of actinobaterium

Out of the 9 strains (BKM 1-9) which showed cellulase activity, the potential actinobacterial strain, BKM 9 was identified based on the microscopic, cultural, physiological and chemotaxonomical charecteristics (International Streptomyces Project). Results showed that the cell wall of the strain possessesd LL-DAP and glycine. Presence of LL-DAP along with glycine indicated the cell wall chemo type I (Lechevalier and Lechevalier, 1970). It is important to note that the presence of spores in a long chain occurring on the aerial mycelium eliminates all the other genera having the cell wall type – I except *Streptomyces* (Lechevalier and Lechevalier, 1970). The branched nature of the substrate mycelium clearly indicated that the strain BKM 9 belongs to the genus *Streptomyces*.

Aerial mycelial colour of the strain was grey with the presence of reverse side pigment. Spore chain morphology revealed that the strain possesses Retinaculiaperti spore chain and smooth spore surface (Fig.1). When the results (cultural, morphological, philological and biochemical characteristics) obtained for the strain BKM 9 were compared with those of the *Streptomyces* reference species (S*treptomyces minoensis*) given in the key of Nonomura (1974) (Table 1). BKM 9 showed variation only in one character i.e. negative utilization of arabinose. Except this, all the other characters were similar to those of S. *minoensis* and hence the strain BKM 9 was identified as a species close to *S. minoensis.*

Screening for cellulase producing actinobacterium

Ability to produce a variety of cellulolytic enzymes is a well known phenomenon in terrestrial mesophillic actinobacteria and reports are available on the cellulolytic enzymes isolated from marine actinobacteria. In the present study, 9 strains showed cellulase activity. Of these, only one strain, BKM 9 showed higher enzyme activity (Fig.2). Chandramohan *et al.* (1972) found higher cellulase activity in *Streptomyces* sp. isolated from fish nets and sail clothes. Murugan *et al.* (2007) studied the cellulolytic activity of *Streptomyces* sp. isolated from the estuarine fin fish *Muguil cephulis,* and reported the maximum activity at pH 8 at 35°C and found that there are differences in the quantities of enzyme production. A total of twenty nine strains were isolated from the Kodiyakarai coastal sediments of the Bay of Bengal and ten of them exhibited multiple enzyme activity. Of these, the best one (GK-22) was selected based on zone formation for amylase, cellulase and protease and its growth conditions were standardized for better production of multiple enzymes (Manivasagam *et al*., 2010).

Selvam *et al*. (2011) screened nine actinobacterial isolates, out of which, three strains showed higher amylolytic, cellulolytic and lipolytic activity i.e 6.48, 8.93 and 700 IU/ml respectively. Soil dwelling actinobacteria collected from the Coringa mangroves, Andhra Pradesh also exhibited amylase, cellulase, L-asparaginase, chitinase and protease activities (Deepthi *et al*., 2012). Production of hydrolytic enzymes, amylase, cellulase and lipase revealed that the mangrove associated actinobactera would serve as a good source for halo tolerant degrading enzymes especially cellulase and lipase (Reyad, 2013). Das *et al*. (2014) have also opined that the actinobacteria are efficient degraders of plant debris of the Valapattanam mangroves of Kerala as they produce extracellular enzymes like cellulase, xylanase and pectinase. It is worth mentioning here that among the 55 isolates from the Coring mangroves, 28 were potential producers of commercially important enzymes such as L-asparaginase, cellulase and amylase (Mangamuri *et al*., 2014). Thus, these studies, including the present one, reveal that the mangrove habitat, containing rich sources of nutrients and organic matter, might provide with suitable niches for actinobacteria.

Optimization of growth parameters and characterization of cellulase production

pH

pH is one of the most important growth factors that determines the growth and morphology of microorganisms as they are more sensitive to the hydrogen ion concentration of the medium. In the present study, BKM 9 (*Streptomyces sp*.) was active at a pH range of 5-9 with the maximum activity (12.5 IU/ml), at pH 8 (Fig.3). This is in agreement with the study of Solingen *et al.* (2001) who isolated *Streptomyces* sp. from the east African soda lake with an optimal pH 8, suggesting the importance of alkaline nature of the habitat of the actinobacteria for enzyme production. Similarly, Nakai *et al*. (1987) isolated *Streptomyces* sp. whose optimal enzyme activity was at pH 8.5. Maximum activity of cellulase (88 IU/ml), was also obtained at pH, 7.0 and temperature, 45°C (Manivasagam *et al*., 2010). But, most cellulases which are commercially available showed optimum activity in a pH range of 4 to 6 and are usually inactive at alkaline pH. In contrast, production of several cellulases in alkaline optimum pH, like that of the present study, has also been found in the

alkalophilic *Bacillus* strains of industrial importance (Au and Chan, 1987).

Temperature

Optimum temperature for microbial enzyme production depends whether an organism is mesophilic or thermophilic. Higher cellulase production in BKM 9 (*Streptomyces sp*.) occurred at 37°C (14 IU/ml), whereas it decreased at higher temperatures, in the present study (Fig. 4), envisaging that the optimal production of the enzyme occurred at mesophilic temperature. Lower enzyme production at higher temperatures could be due to the denaturation of enzymes (Haq *et al*., 2006). Murugan *et al.* (2007) noticed higher cellulase production at 35°C in *Streptomyces* sp., isolated from the esturine fish. Abdel-Shakour and Roushdy (2009) have also reported higher amount of cellulase production at 35°C in *Streptomyces* sp. isolated from the mangrove sediments of the Hurgada natural protectorates in Egypt.

Carbon sources

In the present study, culture media were supplemented with the readily available carbon sources (galactose, fructose, maltose, sucrose, glycerol and glucose), in addition to CMC and without carbon sources, which served as control. Presence of carbon sources with CMC increased cellulase production in BKM 9 (*Streptomyces sp*.). Presence of maltose in the production medium enhanced the cellulase activity (16.8 IU/ml), which was higher than that of the control (14.2 IU/ml) (Fig. 5). In BKM 9 (*Streptomyces sp*.) all the carbon sources enhanced the cellulase activity as compared to that of the control. This lends support to the study of Merivorie *et al.* (1984), who recorded good growth of *Streptomyces* in easily metabolisable carbohydrates like glucose, glycerol and fructose.

Nitrogen source

Effect of nitrogen sources (peptone, yeast extract and beef extract) was studied on the production medium by keeping the optimum pH, temperatutre and carbon sources at constant level. Among the different nitrogen sources, yeast extract was the best for the cellulase activity followed by beef extract and peptone (Fig. 6). Similarly, higher yield of cellulase was obtained in *Streptomyces drozdowiczii* (595 U/l) in a medium supplemented with yeast extract (Grigorevski de Limaa *et al*., 2005). But, Alam *et al.* (2004) found that a higher cellulase activity was presented in the beef extract. Maintaining the physical factors and the carbon sources at optimized condition, nitrogen sources of varying nature were tested by Manivasagam *et al*. (2010) and the test strain showed higher activity in the beef extract.

Amino acids

As amino acids are the building blocks of protein, they have a good influence on the cellulase synthesis by *Streptomyces* (Vyas *et al*., 2005). Among the different amino acids (L-glutamine, L-asparagine, L-phenylalanine, L-tyrosine and L-histidine) added to the production medium (0.1% W/V), phenyl alanine (19.2 IU/ml) and glutamine (18.9 IU/ml) enhanced the cellulase activity of BKM 9 (*Streptomyces* sp.) followed by asparagine, histidine and tyrosine (Fig.7). Szajer (1975) has also reported that the cellulase activity of *Fusarium* was enhanced by the addition of aminoacids. Kundu (2006) observed maximum enzyme activity in L-Phenylalanine in *Streptomyces galilaeus*. When different amino acids were tested, amylase and cellulase enzyme production was higher in L-asparagine and minimum in L-histidine (Manivasagam *et al*., 2010).

Purification of cellulase

Cellulase, produced was purified by a three-step strategy including ammonium sulfate precipitation, dialysis and ion-exchange chromatography. Recovery of enzyme by ammonium sulfate precipitation was 79.9% followed by dialysis (46.7%) and ion-exchange chromatography (39.96%) (Table 2). The overall purification attained 17.7 fold purity with a specific activity of 2,466.67 IU/mg. In a previous study, purification of cellulase derived from *Thermomonospora* gave 17.1 fold purity with a specific activity of 237.69 IU/mg (George *et al*., 2001). Molecular mass of the enzyme estimated from SDS - PAGE was 40 kDa. This is in the range of some other low molecular mass endoglucanases (23–42 kDa) produced by *Bacillus* sp. (Au and Chan, 1987; Sharma *et al*., 1990). In another study, enzyme was purified to 12.5-fold with a 37% yield and a specific activity of 2,548.75 IU/mg. The purified enzyme displayed maximum activity at the mesophilic temperature (27–35°C) and a broad pH range with an optimal activity at pH 9.0 (Shanmughapriya *et al*., 2009).

A wide range of bacteria are known for their production of hydrolytic enzymes and among them, streptomycetes are the best known enzyme producers (Vinogradova and Kushnir, 2003; Sahu *et al*., 2007). They are capable of secreting an array of extracellular enzymes including cellulases, chitinases and xylanases (Murugan *et al*., 2007; Aarthi *et al*., 2009). Ability to secrete a large amount of extracellular enzymes is characteristic of certain actinobacteria and such organisms are more suited for the production of higher levels of extracellular cellulases. Most commonly studied cellulolytic actinobacteria include *Streptomyces*, *Thermomonospora* and *Actinomucor* (Sivakumar *et al*., 2005). Further, these cellulase producers have attracted considerable research interest due to their potential application in the recovery of fermentable sugars from cellulose, for human consumption (Jang and cheng, 2003), as compared to

Table 1. Comparison between the strain BKM 9 and *Streptomyces minoensis*.

Character studied (as per Nonomura key)	Strain BKM-9	*Streptomyces minoensis*
Colour of aerial mycelium	Gray	Gray
Melanoid pigment	-	-
Reverse side pigment	+	+
Soluble pigment	-	-
Spore chain	Retinaculiaperti	Retinaculiaperti
Spore surface	Smooth	Smooth
Carbon source assimilation		
Arabinose	-	+
Xylose	+	+
Inositol	+	+
Mannitol	+	+
Fructose	+	+
Rhamnose	+	+
Sucrose	+	+
Raffinose	+	+

Table 2. Purification of cellulase produced BKM 9.

Purification steps	Total activity (IU/ml)	Total protein (mg)	Specific activity (IU/mg)	Fold of purification	Yield %
Culture extract	111.1	0.819	138.87	1	-
Ammonium sulphate precipitate	88.8	0.145	612.41	4.42	79.9
Dialysis	67	0.087	743.3	5.29	46.7
Sephadex G100 (Ion-exchange chromatography)	44.4	0.018	2466.67	17.7	39.96

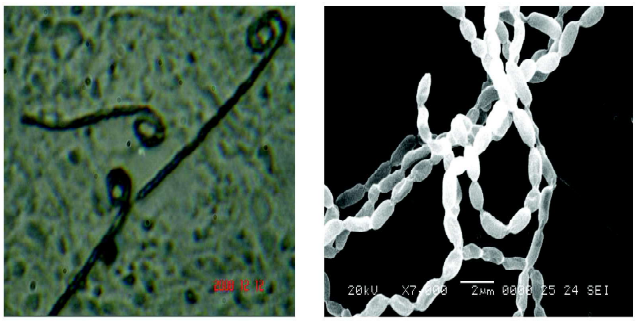

Fig.1. Retinaculiaperti spore chain and smooth spore surface of BKM 9.

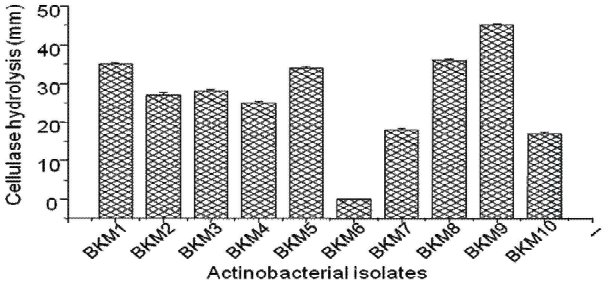

Fig. 2. Screening of actinobacterial strains for cellulase production.

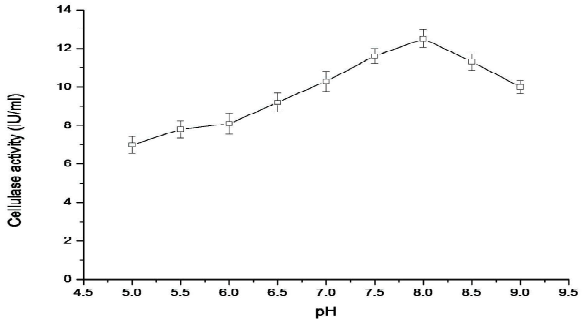

Fig. 3. Effect of pH on cellulase production by BKM 9.

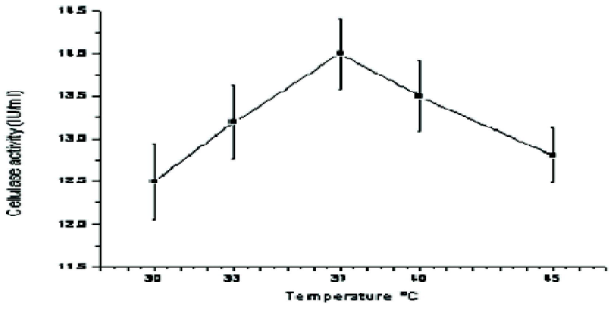

Fig. 4. Effect of temperature on cellulase production by BKM 9.

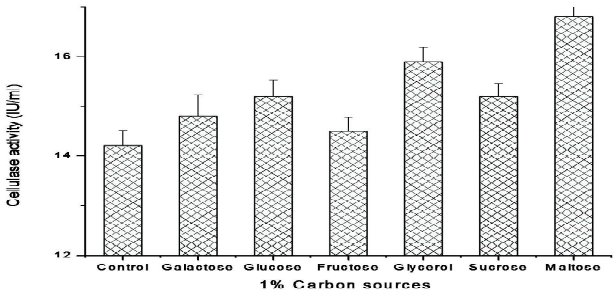

Fig. 5. Effect of carbon sources on cellulase production by BKM 9.

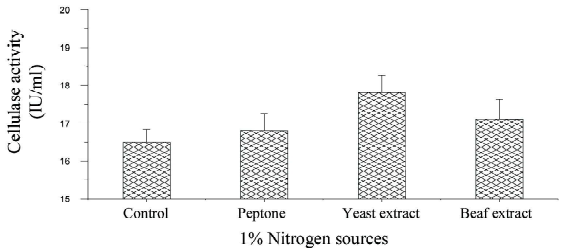

Fig. 6. Effect of nitrogen sources on cellulase production by BKM 9.

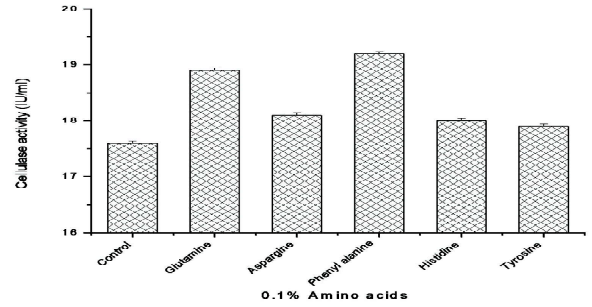

Fig. 7. Effect of amino acids on cellulase production by BKM 9.

the anaerobic cellulase production by *Paenibacillus curdlanolyticus* (Pason *et al*., 2006).

It is worth mentioning here that Abdel-Shakour and Roushdy (2009) have found that *Streptomyces* sp. H-1 produced a very powerful cellulase enzyme system capable of degrading natural cellulosic biomass residues and exhibited higher enzymatic activity at the specified factor, which reached 95.00 IU/mL^{-1}. Partially purified enzyme increased the activity up to 180 IU/mL^{-1}. Molecular weight determination revealed the presence of two distinct bands of about 81 and 43 KDa.

CONCLUSION

Biological aspects of processing the cellulosic biomass involving cellulases and cellulolytic microorganisms have become the crux of the researchers (Sukumaran *et al*., 2005). Present findings have revealed the fact that the factors such as pH, temperature corban and nitrogen sources and amino acids significantly effect the microbial cellulase production. It should be borne in mind that the cellulase enzymes from actinobacteria can strengthen the microbial cellulase sources in addition to fungal and bacterial cellulases for human and industrial uses. Hence, further investigations are required to fully utilize the potential of the microbes especially marine actinobacteria for cellulase production using the modern protein engineering technology.

ACKNOWLEDGEMENT

Authors thank the Director and Dean, Centre of Advanced Study in Marine Biology and the authorities of Annamalai University for providing with necessary facilities. They thank Prof. L. Kannan, Former Vice chancellor, Thiruvalluvar University, for critically going through the manuscript and offering suggestions. Dr.J.Rajkumar thanks the Ministry of Human Resource Development (MHRD) and Dr.K.Sivkumar thanks DBT, GOI for financial support.

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