African Journal of Biotechnology Vol. 11(5), pp. 1185-1196, 16 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1819
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Optimization of process parameters for the production of alkali-tolerant carboxymethyl cellulase by newly isolated *Streptomyces* sp. strain NEAE-D

Noura El-Ahmady El-Naggar[1]* and Nayera A.M. Abdelwahed[2]

[1]Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, City for Scientific Research and Technology Applications, Alexandria, Egypt.
[2]Department of Chemistry of Natural and Microbial Products, National Research Center, 12311 Dokki, Cairo, Egypt.

Accepted 8 August, 2011

One hundred and forty one actinomycetes strains were isolated from Egyptian and Saudi Arabia soils and decayed rice straw samples. The isolated actinomycetes strains were screened for their cellulolytic activities on carboxymethyl cellulose (CMC) - containing agar plates. *Streptomyces* sp. NEAE-D showed the highest carboxymethyl cellulase (CMCase) activity. The optimum conditions for CMCase production were the use of pretreated rice straw as the best carbon source that supported better CMCase production than carboxymethyl cellulose after 5 days incubation at pH 6.5 and at 35°C in the presence of yeast extract as nitrogen source. Crude CMCase activity was characterized. The highest activity was recorded after 50 min incubation, at 50°C, pH 6.0 and 2% substrate concentration, the CMCase was shown to be alkali-tolerant enzyme whose activity was stable over a broad pH range (7 to 10). The enzyme retained 97.31 and 70.99% of its activity at 50 and 60°C respectively after 60 min. High performance liquid chromatography (HPLC) analysis of the hydrolysate of saccharified pretreated rice straw identified the formation of glucose, cellobiose, mannose and xylose as byproducts.

Key words: Carboxymethyl cellulase, production, pretreated rice straw, alkali-tolerant, *Streptomyces* sp. NEAE-D, characterization.

INTRODUCTION

Carboxymethyl cellulase (CMCase), one of the members of cellulase complex, cleaves the internal glycosidic bonds of cellulosic chains and acts synergistically with exoglucanases and β -glucosidases during the solublization of cellulosic material. It is an industrially important enzyme. CMCases have great potential for its utilization in the food industry; for coffee processing, extraction and clarification of juices (Galante et al., 1998). It is widely used in textile industry; modify the surface properties of cellulosic fibers and fabric in order to achieve a desired surface effect (Kotchoni et al., 2003) ; used in laundry detergents; in the pulp and paper industry for various purposes (Ogel et al., 2001); facilitates fermentation of biomass into biofuels and even to treat phytobezoars, a form of cellulose bezoar found in human stomach.

Screening and evaluation of nutritional and environmental requirements of microorganism is an important step for bioprocess development. CMCase activities are altered with varying pH and temperature and characterization of produced enzyme require knowledge about optimum pH, temperature stability and substrate specificity. Therefore, the enzyme is characterized to find out the best level of performance for enhanced efficiency of the process (Bhat, 2000; Parry et al., 2002).

However, the relatively high cost of enzyme production has hindered the industrial applications of the enzymatic process (Xia and Len 1999). Therefore, the use of abundantly available and cost-effective agricultural by-products, such as rice straw to achieve higher cellulase yields allows reduction of the overall cost of enzyme production.

Actinomycetes are well known for their ability to de-

*Corresponding author. E-mail: nouraelahmady@yahoo.com. Tel: (002)0103738444. Fax: (002)03 4593423.

compose complex molecules, particularly lignocellulose components, which make them important agents in the decomposition processes (Lacey, 1997). Additionally, the apparent widespread ability of actinomycetes to generate soluble ligno-carbohydrate from straw has been confirmed (Ball et al., 1990; Mason et al., 2001).

The present study aimed to describe the isolation of actinomycetes strains, which have cellulolytic activities, from Egyptian and Saudi Arabia soils, to maximize CMCase production by optimizing fermentation parameters using rice straw as substrate in order to reduce the cost of production as well as to investigate the characterization of alkali-tolerant CMCase from *Streptomyces* sp. NEAE-D.

MATERIALS AND METHODS

Collection of lignocellulose samples

Different native lignocelluloses like rice straw and sugarcane bagasse were collected from local fields of Dakahliyah governorate; rice husk, oat meal, wheat bran and saw dust were purchased from the local market, Egypt. Then, they were oven-dried at 65°C for two days to reduce the moisture content and were milled to mesh size powder. The powder of each raw material was used as carbon source.

Isolation of actinomycetes

Streptomyces spp. used in this study were isolated from various soil samples collected from different localities of Egypt [Dakahliyah governorate ,Damietta governorate, north western coast of Egypt from New Borg El Arab to El Saloum, Janaklis(Beheira) and Kafr El-Sheikh] and Saudi Arabia. Actinomycetes from the soils were isolated using standard dilution plate method procedure on starch nitrate agar plates; then plates were incubated for a period of seven days at 30°C. Mycostatin was incorporated as an antifungal agent to inhibit the fungal growth in the isolation medium. Colonies of actinomycetes were selected and isolated. *Streptomyces* isolates (a total of 141 morphologically different isolates) were purified and maintained as spore suspensions in 20% (v/v) glycerol at -20°C for subsequent investigation (Hopkins et al., 1985). The medium used for isolation, cultivation and stock maintenance of isolated strains was starch nitrate agar medium (Waksman, 1959). It contained (g/L): soluble starch, 10; KNO_3, 2; K_2HPO_4, 1; NaCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; $CaCO_3$, 3; agar, 20 and distilled water up to 1L.

Plate screening for caboxymethylcellulase activity

For plate screening, caboxymethylcellulose-agar medium was used. This medium consisted of (g/L): CMC,10.0; $NaNO_3$, 1.2 ; KH_2PO_4, 3.0 ;K_2HPO_4, 6.0; $MgSO_4 \cdot 7H_2O$, 0.2 ; $CaCl_2$, 0.05 ; $MnSO_4$.$7H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.001; yeast extract , 0.5; agar, 20.0 at pH 7.0. Agar plates were prepared and spot inoculated with spores and incubated at 30°C for six days followed by 18 h at 50°C (Onsori et al., 2005) to accelerate the action of extracellular cellulases and thus rapidly develop clear zone around the cellulase-producing colonies. For cellulolytic activity observations, plates were stained with 0.1% (w/v) Congo red dye for 15 min followed by destaining with 1 M NaCl solution for 15 to 20 min (Apun et al., 2000); unstained areas (clear zone of hydrolysis) indicate where the CMC was hydrolysed. The diameter of the clear zone can be measured to provide a quantitative comparison of cellulolytic activity. The strain which showed the most promising result was selected for further experiments.

CMCase assay

CMCase activity in the culture filtrate was determined according to Miller (1959). A reaction mixture of 0.25 ml of 1% (w/v) carboxymethyl cellulose (CMC sodium salt, low viscosity, Sigma Chemical Co., St. Louis, MO, USA) solution in MCIlvaine buffer (pH 6), and 0.25 ml of the culture filtrate was incubated at 45°C for 40 min (blanks were prepared in the same way and boiled for 5 min). After incubation, the enzyme activity was stopped by adding 1.5 ml DNS-reagent; tubes were placed in a boiling water bath for 15 min and then cooled to room temperature. The O.D. of the samples was immediately measured at 540 nm. CMCase activity was expressed in terms of units. One unit of carboxymethyl cellulase activity was defined as the amount of enzyme required to release 1 µmole reducing sugars per minute per ml under the assay conditions.

Dilute-acid pretreatment of rice straw

Rice straw was pretreated according to the modified method of Zhu (2005). Rice straw was grinded to achieve more uniform particle size distribution (1 cm). 300 ml of 1% H_2SO4 was added to 15 g of the grinded rice straw in a 500 ml wide-mouth Pyrex bottle and the slurry was stirred with a glass rod for thorough mixing. The slurry was pretreated at 121°C in an autoclave for 1 h. After pretreatment, the Pyrex bottle was allowed to cool before opening. The biomass was repeatedly washed with distilled water until the supernatant pH reached 6.0. The biomass was dried at 45°C in an oven for three days. The dried biomass was ground to ensure more uniform particle size distribution.

Submerged fermentation conditions

Submerged fermentation for enzyme production was carried out in Erlenmeyer flasks (250 ml) containing 50 ml of the production medium containing (g/L): rice straw as substrate, 10 to15; $NaNO_3$, 1.2; KH_2PO_4, 3; K_2HPO4, 6.0; $MgSO4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.05; $MnSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.001; yeast extract, 0.5 and distilled water up to 1L. The pH was adjusted to 7.0. The flasks were autoclaved at 121°C for 20 min, inoculated with three agar disks of 9 mm diameter taken from the seven days old stock culture according to the method of Gill et al. (2003) and incubated at 30°C for six days on a rotary shaker at 200 rpm. After incubation period, the cells were removed by centrifugation; the mycelium free supernatant was used as crude enzyme preparation for estimation of CMCase activity.

Optimization of fermentation conditions for CMCase production

Different process parameters influencing the enzyme production by *Streptomyces* sp. NEAE-D under submerged fermentation conditions were studied for maximum enzyme production as follows: different types of carbon sources including; inulin, starch, xylan, cellobiose, cellulose powder, carboxymethyl cellulose, rice straw, pretreated rice straw, wheat bran, oat meal, sugar cane bagasse, saw dust and rice husk were separately added as a sole carbon source to the culture medium in the concentration of 1% (w/v). Media were inoculated and incubated at 30°C for six days with shaking at 200 rpm.

Figure 1. Cellulolytic activity of *Streptomyces* sp. NEAE - D on cellulose agar indicated by clearing zone surrounding the colony.

To investigate the effect of substrate concentration on yield of CMCase, rice straw and cellulose powder were added to the basal salt media as a carbon source at different concentrations (0. 5 % to 2%; w/v).

The effect of incubation periods was evaluated at 24 h intervals by checking the enzyme activity. The effect of pH value on the production of CMCase was studied at various pH values ranging from 4.0 to 9.0. The effect of temperature on the production of CMCase was determined at various temperatures ranging from 20 to 50°C at pH 6.5. The effect of supplementation of nitrogen sources was estimated using ammonium chloride, ammonium phosphate, potassium nitrate, sodium nitrate, urea, yeast extract, peptone and casein which were added on equivalent nitrogen bases.

Surfactants including; Tween 20, Tween 40 and, Tween 80 were added to the cultivation media which contained pretreated rice straw at a concentration of 0.1%. Control was prepared in the same way without surfactants. The media were inoculated and incubated at 30°C for five days with shaking at 200 rpm.

Physicochemical properties of the enzyme

Effect of temperature, incubation period and pH

The enzyme activity was measured at different incubation intervals (10 to100 min). Effect of temperature on the enzyme activity was estimated by incubating reaction mixtures for the optimum incubation time with increasing temperatures from 25 to 70°C .The effect of pH on enzyme activity was studied by incubating the enzyme with substrate in the presence of buffers (MCIlvaine buffer (pH 3 to 8) and Glycine - NaOH (pH 8.6 to10.6). pH values were adjusted to cover a range from pH 3 to 10.

Effect of different substrate concentrations

Effect of different substrate concentrations on the enzyme activity was examined using different concentrations of the substrate ranging from 0.50 to 5.0 % under optimum conditions.

Thermostability and pH stability

For studying enzyme thermostability, enzyme solution was subjected separately to various temperatures (40 to 70°C) for various time periods (15 to 120 min) then the remaining activity was assayed. With respect to pH stability, enzyme was dissolved in various buffers. pH values were adjusted to cover the range from pH 3 to 10 and incubated at 30°C for 24 h with 6 h intervals. The residual activity for the enzyme after each pH treatment was determined after adjusting the pH to the optimum one.

Estimation of total proteins

Total protein in culture filtrate was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

HPLC

The hydrolysate of saccharified pretreated rice straw was subjected to determination of free sugars using HPLC (Agilent 1100 HPLC system) with an APS-2 Hypersil column (150 × 4.6 mm, particle size 3 µm) using a mobile phase of sodium dihydrogen phosphate buffer and acetonitrile (0.253 g of sodium dihydrogen phosphate monohydrate in 190 ml of water and mix with 810 ml acetonitrile); the solvent flow rate was 2 ml/min and column temperature was 38°C. Injection volume of samples used for HPLC analysis was 20 µl of the hydrolysate. The soluble sugars were quantified by reference to the peak areas and retention times obtained for the standards for glucose, xylose, arabinose, galactose, cellobiose and mannose that was analyzed in the concentration of 2 mg/ml.

RESULTS AND DISCUSSION

One hundred and forty one morphologically different actinomycetes isolates were recovered from 35 samples collected from different localities (32 soils and decayed rice straw samples from Egypt) and (three soil samples from Saudi Arabia). Actinomycetes strains were examined for cellulolytic activity on CMC- containing agar plates (Figure 1). *Streptomyces* NEAE-D showed wider clear zone diameter around its colonies. The cellulolytic activity of the selected strain was confirmed under submerged fermentation indicating the highest cellulose degradation and therefore was selected for the CMCase production.

On the basis of morphological, cultural, physiological and chemotaxonomic properties, together with 16S rRNA sequence and sequencing product that was deposited in the GenBank database under accession number JF906306 (data not shown), it is evident that isolate NEAE-D belongs to the genus *Streptomyces* and was identified as *Streptomyces* sp. NEAE-D.

Comparison of effect of different lignocelluloses and other carbon sources on CMCase production

Pure substrates are too expensive to be employed for industrial production of cellulases, so alternative substrates, particularly crude raw materials of agricultural

Table 1. Effect of different carbon sources on reducing sugars level, saccharification (%) and CMCase production by *Streptomyces* sp. NEAE-D.

Carbon source	CMCase activity (U/ml)	Specific activity (U/mg protein)	Reducing sugar (mg/ml)	Saccharification (%)
Carboxymethyl cellulose	34.179	66.366	1.230	12.301
Cellulose	43.594	51.975	1.569	15.689
Rice straw	30.658	48.518	1.103	11.034
Pre-treated rice straw	45.766	64.733	1.647	16.471
Wheat bran	21.082	28.840	0.759	7.587
Rice husk	40.253	46.714	1.449	14.49
Oat meal	28.524	48.842	1.027	10.266
Sugar cane bagasse	25.272	33.209	0.910	9.095
Saw dust	41.019	42.863	1.476	14.763
Cellobiose	12.621	14.942	0.454	4.542
Inulin	14.514	19.668	0.522	5.224
Starch	22.568	15.738	0.812	8.122
Xylan	26.564	37.949	0.956	9.560

Table 2. Effect of different carbon source concentrations on reducing sugars level, saccharification (%) and CMCase production by *Streptomyces* sp. NEAE-D.

Carbon source concentration (%)	CMCase activity (U/ml)	Specific activity (U/mg protein)	Reducing sugar (mg/ml)	Saccharification (%)
Cellulose				
0.5	16.618	21.088	0.598	5.981
1	42.125	41.890	1.516	15.161
1.5	41.680	53.095	1.500	15.001
2	36.872	60.844	1.327	13.270
Pre-treated rice straw				
0.5	24.792	32.072	0.892	8.923
1	44.029	48.346	1.585	15.846
1.5	49.169	63.386	1.770	17.696
2	35.009	28.210	1.260	12.600

origin, have been explored as cost-effective substrates. Carboxymethyl cellulose of the production medium was replaced with alternative substrates such as rice straw, pre-treated rice straw, wheat bran, rice husk, oat meal, sugar cane bagasse, saw dust and other different carbon sources (cellulose powder, cellobiose, xylan, starch and inulin) which were evaluated as substrate for CMCase pro-duction and compared with carboxymethyl cellulose as control and are presented in Table 1. The results indicate that CMCase production from *Streptomyces* sp. NEAE-D was constitutive in nature. Among these, xylan, starch, inulin, wheat bran, oat meal, sugar cane bagasse and cellobiose had no significant stimulating effect on CMCase production by *Streptomyces* sp. NEAE-D while cellulose powder, pre-treated rice straw, saw dust and rice husk significantly enhanced CMCase production by this isolate as compared to that on pure CMC. The maximum CMCase yield was supported by pre-treated rice straw (45.766 U/mL) and cellulose powder (43.594U/ml) while the minimum production was recorded in the presence of cellobiose as carbon source (12.621 U/ml). Different concentrations of pre-treated rice straw and cellulose powder (0.5, 1.0, 1.5 and 2%; w/v) were added to the cultivation medium. The maximum CMCase yield was obtained with 1.5% of pre-treated rice straw (Table 2). Therefore, further experiments were conducted to optimize the CMCase production by *Streptomyces* sp. NEAE-D by using 1.5% pretreated rice straw as sole carbon source in the CMCase production medium. The cellulase production by *Trichoderma harzianum* MTCC 8230 was studied using rice straw as substrate that supported better production than

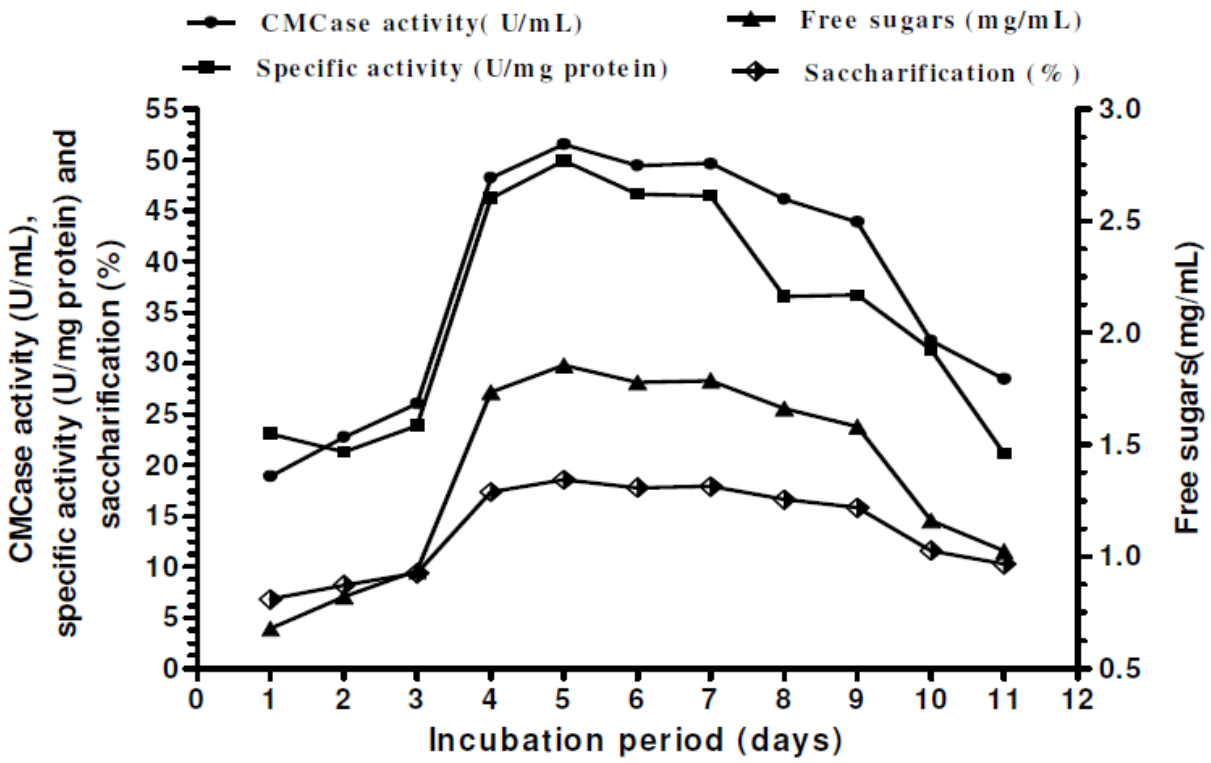

Figure 2. Effect of incubation periods on reducing sugars level, saccharification (%) and CMCase production by *Streptomyces* sp. NEAE-D**.**

carboxymethyl cellulose (Kocher et al., 2008).

Effect of incubation periods on CMCase production

Streptomyces sp. NEAE-D with potential CMCase production was subjected to produce CMCase in liquid culture and the effect of incubation time on enzyme yield is depicted in Figure 2. Maximum CMCase activity was obtained after five days incubation; similar results were reported by Semedo et al. (2000) , El-Naggar et al.(2011) and Jang and Chen (2003). The decrease in CMCase activity shown by *Streptomyces* sp. NEAE-D after attaining its maximum peak period of enzyme secretion could be attributed to catabolic repression of byproducts of cellulases action on rice straw (cellobiose and glucose, etc) which inhibit enzyme activity. Depletion of carbon and nitrogen sources causes starvation and hence the organism may not grow and cellulase activity is growth related as reported by Dosoretz et al. (1990).

Effect of pH on CMCase production

The effect of pH-value on CMCase production by *Streptomyces* sp. NEAE-D was examined at various pH values ranging from 4.0 to 9.0 as shown in Figure 3. The enzyme showed high activity at a broad range of pH values (pH 5 to 9) with optimal pH of 6.5. The enzyme production had only about 30.79% decreases at pH 9. *Streptomyces omiyaensis* showed heavy growth and high cellulase activity at pH 6.5 (Alam et al., 2004). Harchand and Singh (1997) also investigated a profound effect of pH on cellulase activity of *S. albaduncus*. Similarly, *Streptomyces* sp. F2621 (Tuncer et al., 2004) and *S. albogriseous* (Van Zyl, 1985) also exhibited maximum endoglucanase activity at initial pH of 6.5 to 7.0.

However, the results appeared to contradict previous results reported by Theberge et al. (1992) who showed that the optimum pH for endo-glucanase from a strain of *Streptomyces lividans* was 5.5. Total cellulase activities of *Streptomyces* sp. were maximal at pH 5.5; CMCase at 5.0 (Okeke and Paterson, 1992).

Effect of temperature on CMCase production

The effect of temperature on the production of CMCase was determined at various temperatures ranging from 20 to 50°C at pH 6.5 (Figure 4). The enzyme showed a good production between 20 to 40°C with maximum activity at 35°C. *Streptomyces* sp. F2621 (Tuncer et al., 2004) and *S. albogriseous* (Van Zyl, 1985) exhibited maximum endoglucanase activity at 26 to 30°C.

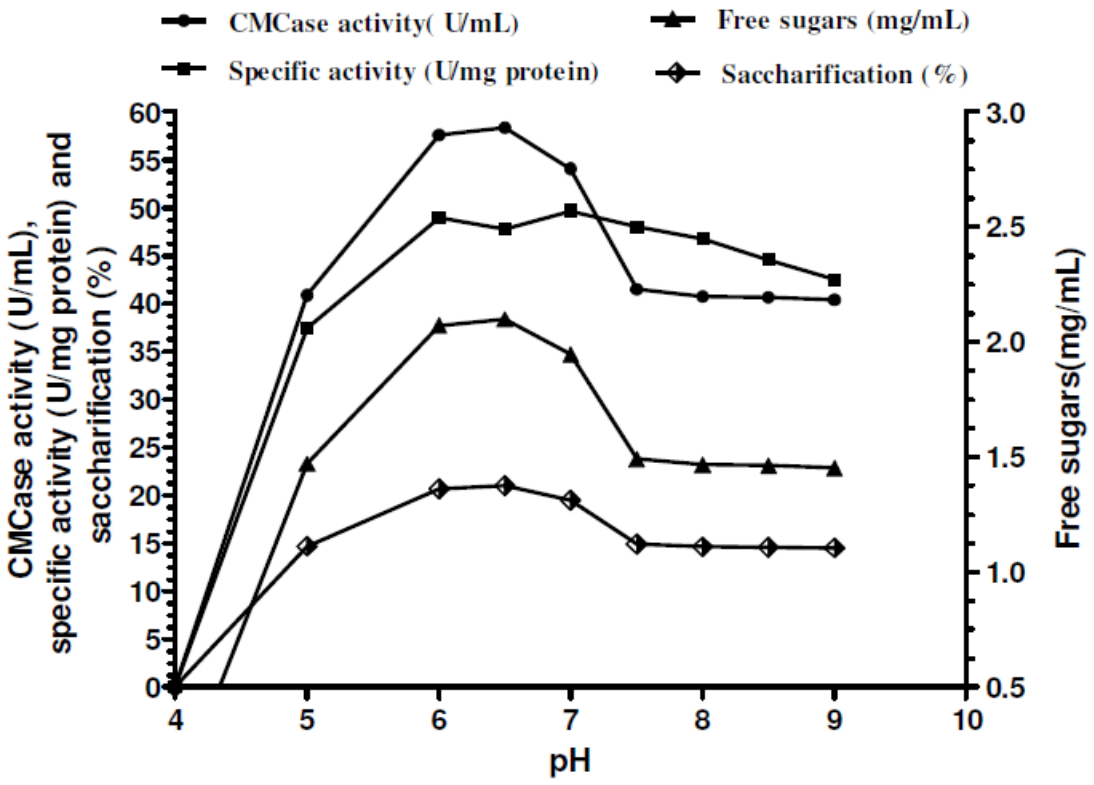

Figure 3. Effect of pH on reducing sugars level, saccharification (%) and CMCase production by *Streptomyces* sp. NEAE-D

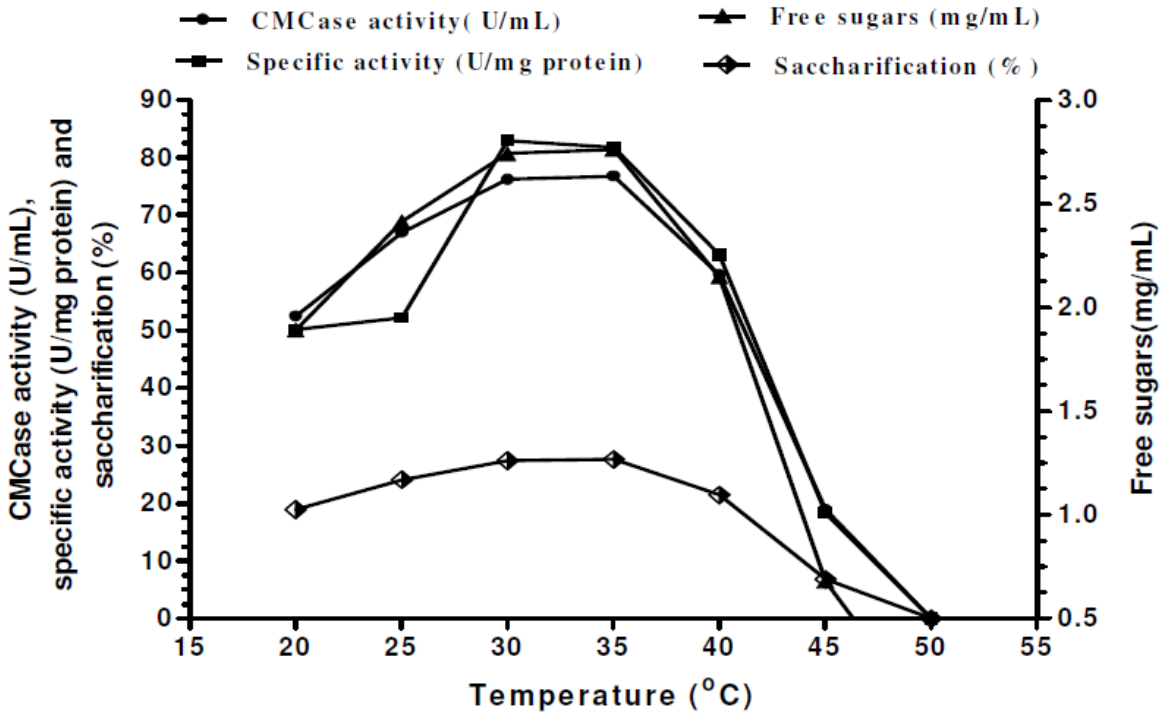

Figure 4. Effect of temperature on reducing sugars level, saccharification (%) and CMCase production by *Streptomyces* sp. NEAE-D.

Table 3. Effect of different nitrogen sources on reducing sugars level, saccharification (%) and CMCase production by *Streptomyces* sp. NEAE-D.

Nitrogen source	CMCase activity (U/ml)	Specific activity (U/mg protein)	Reducing sugars (mg/ml)	Saccharification (%)
$NaNO_3$ + Yeast extract	86.811	121.926	3.124	31.243
NH_4Cl	51.398	99.222	1.850	18.498
$NH_4H_2PO_4$	25.117	60.089	0.904	9.040
KNO_3	53.020	108.206	1.908	19.082
$NaNO_3$	33.726	85.167	1.214	12.138
Urea	19.417	38.073	0.699	6.988
Yeast extract	89.664	135.240	3.227	32.270
Peptone	85.936	119.887	3.093	30.928
Casein	87.338	123.011	3.143	31.433

Table 4. Effect of surfactant on reducing sugars level, saccharification (%) and CMCase production by *Streptomyces* sp. NEAE-D.

Surfactant	CMCase activity (U/ml)	Specific activity (U/mg protein)	Reducing sugars (mg/ml)	Saccharification (%)
Control	84.492	89.423	3.041	30.409
Tween 20	80.858	84.853	2.910	29.101
Tween 40	87.841	99.755	3.161	31.614
Tween 80	103.302	105.763	3.718	37.178

Role of nitrogen sources on enzyme production

The impact of inorganic (ammonium chloride, ammonium phosphate, potassium nitrate, sodium nitrate and urea) and complex nitrogen (yeast extract, peptone and casein) sources was evaluated on CMCase production by *Streptomyces* sp. NEAE-D. Among all the nitrogen sources studied, yeast extract supported the maximum enzyme production (89.664 U/ml) followed by casein (87.338 U/ml) and a combination of yeast extract and sodium nitrate (86.811 U/ml) (Table 3). Yeast extract suited the enhanced production of endoglucanase by *Streptomyces* sp. BRC2 (Chellapandi and Himanshu, 2008). Stutzenberger (1971) verified that the addition of yeast extract (0.1%) to the medium allowed growth and production of cellulases by the actinomycete *Thermomonospora curvata*. Several other investigators have also utilized yeast extract in the composition of culture media for the production of cellulases by actinomycetes (Ishaque and Kluepfel, 1980; Dasilva et al., 1993). Yeast extract was reported as a potential nitrogen source for endoglucanase production by *Streptomyces* sp. F2621 (Tuncer et al., 2004).

Effect of surfactants on CMCase

The maximum CMCase production was achieved (103.302U/ml) when the production medium was supplemented with Tween-80 (Table 4). Additionally, Tween-20 and Tween-40 had no effect on CMCase production. Surfactants are used to increase the permeability of bacterial cell membrane by enhancing membrane transport and excretion of extracellular enzymes into the production media (Okeke and Obi, 1993; Chellapandi and Himanshu, 2008).

Effect of different incubation periods on CMCase activity

The effect of incubation periods on CMCase activity is depicted in Figure 5A. It was observed that CMCase activity gradually increased and reached its maximum (91.713 U/ml) after 50 min; after that enzyme activity slowly decreased. Muthezhilan et al. (2007) reported that 60 min incubation was the optimum for endoglucanase and exoglucanase activities.

Effect of the temperature on CMCase activity

To investigate the optimal incubation temperature for the CMCase activity, the reaction mixtures were incubated in the range of 25 to 70°C. Maximum activity for CMCase was obtained at 50°C (96.257 U/ml) after 50 min, however, considerable decreases were recorded below 35°C or above 65°C (Figure 5B). The enzyme retained60.03% of its maximum activity at 70°C. These results are agreement with the results reported by Jang and Chen (2003) who described a CMCase produced by

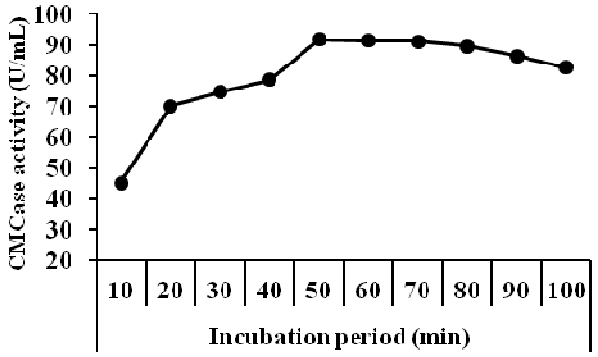

Figure 5A. Effect of incubation periods on CMCase activity.

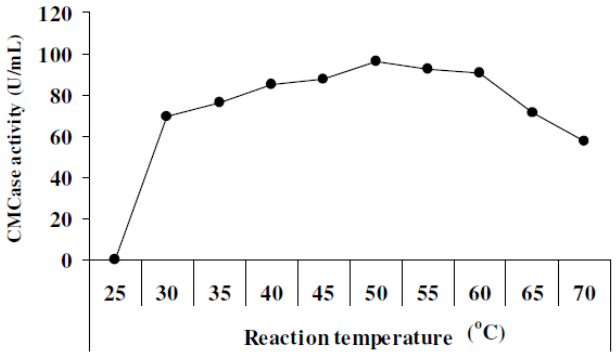

Figure 5B. Effect of reaction temperature on CMCase activity.

a *Streptomyces* T3-1 with optimum temperature of 50°C, whereas Schrempf and Walter (1995) described a CMCase production by a *S. reticuli* at an optimum temperature of 55°C. Wachinger et al. (1989) found *S. reticuli* optima activities of the cellulase system in the range of 45 to 50°C, been extremely sensitive outside of this range. Furthermore, Ishaque and Kluepfel (1980) found *S. flavogriseus* optimum activity at 50°C for CMCase.

Effect of pH on CMCase activity

The effect of pH on enzyme activity was investigated at pH values ranging from 3.0 to 10.0. The effect of pH on CMCase activity was determined under the standard conditions for the CMCase assay (50°C after incubation for 50 min). The enzyme was active over a broad pH range from pH 4 to 9 with maximum activity at pH 6 (Figure 5C). This result is similar to that reported by Dasilva et al. (1993) who isolated a *Streptomyces* sp. producing an extracellular constitutive alkaline CMCase, with optima activities at pH values of 6.0 and 7.0 at 35°C. In addition, Jaradat et al. (2008) showed that CMCase from the active *Streptomyces* isolate J2 was

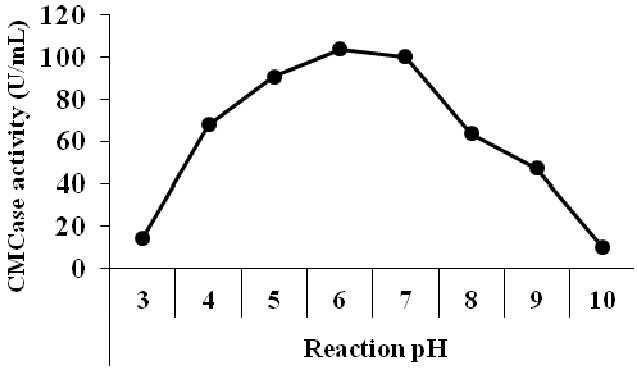

Figure 5C. Effect of reaction pH on CMCase activity.

found to be active over a pH range of 4 to 7 with maximum activity at pH 6 which is considerably similar to that reported by Kluepfel et al. (1986) who reported that the maximal values of *S. lividans* 1826 CM-cellulase activity were obtained at pH 7. Semedo et al. (2000) found optimum pH 7.0 for CMCase activity produced by *S. drozdowiczii*, with an extra peak with minor activity in pH 11.0, whereas Jang and Chen (2003) obtained a CMCase produced by *Streptomyces* T3-1 with optimum activity at pH 7.0 to 8.0. Okeke and Paterson (1992) showed that the optimum pH for total cellulase activities of *Streptomyces* sp. was pH 5.5; CMCase activity was maximal at pH 5.0. Theberge et al. (1992) showed that the optimum pH for endoglucanase from a strain of *S. lividans* was 5.5.

Effect of substrate concentration on CMCase activity

CMCase of *Streptomyces* sp. NEAE-D was evaluated under optimal conditions with varied substrate concentrations ranging from 0.5 to 5.0% (w/v). The results obtained in Figure 5D indicate that CMCase reached its highest activity rate (165.395 U/mL) at 2.0% (w/v) substrate concentration. This result was explained by Dixon and Webb (1971) since they referred that an increase in substrate concentration made more binding sites available for the enzymes to adhere to and the rate at which product formation would be achieved therefore would be faster. In addition, Sherief et al. (2010) reported that the CMCase reached their maximum activity at 2.0% substrate concentration.

Effect of temperature on the stability of CMCase

The stability of the enzyme was studied by incubating the enzyme in the range of 40 to 70°C. The enzyme was thermostable at 40 and 50°C. At 50°C the enzyme retained 100% residual relative activity during the first 30 min, dropping to 97.31% activity at the end of 1 h.

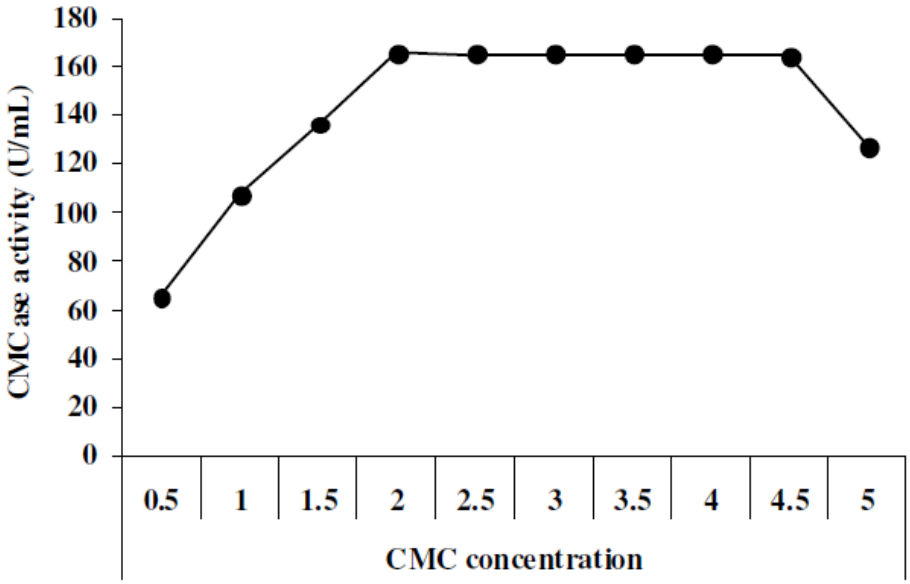

Figure 5D. Effect of substrate concentrations on CMCase activity.

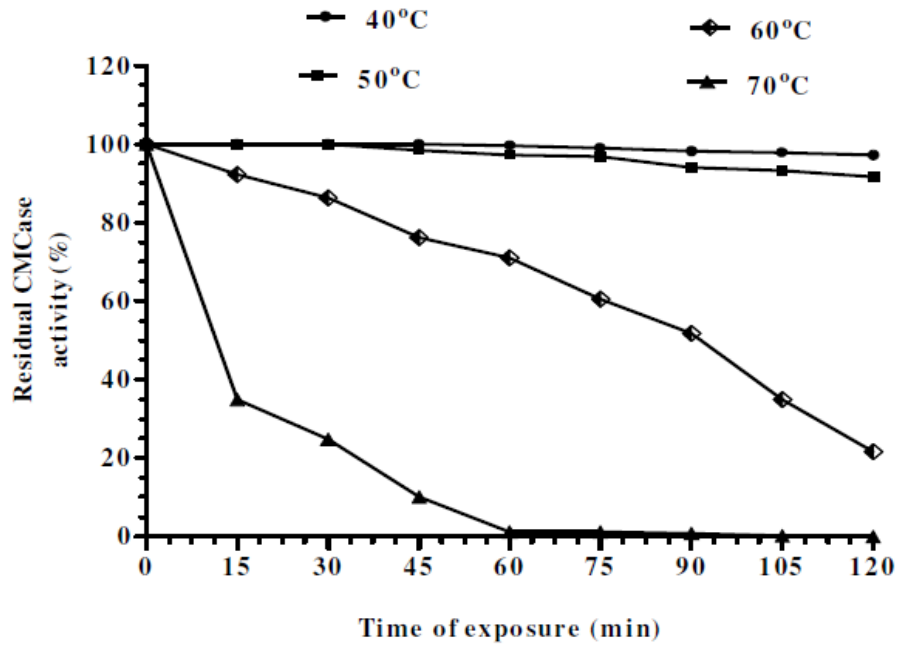

Figure 5E. Thermal stability of CMCase at different temperatures.

Likewise, at 60°C the enzyme activity linearly decreased from 92.215 to 21.673% at 15 min and 2 h, respectively (Figure 5E). This enzyme is more thermally stable than the one reported by Hoshino et al. (1999), who found that 80% residual activity after 30 min incubation at 45°C was found for a *Streptomyces* endoglucanase. However, Lima et al. (2005) found that a crude enzyme from *S. drozdowiczii* was able to retain 100% residual activity at 50°C after 1 h, but only 40% after 2 h.

Effect of pH on the stability of CMCase

The effect of pH on CMCase stability was determined after incubation of the enzyme in various buffers (pH 3 to pH 10) at 30°C for 24 h with 6 h intervals. The residual

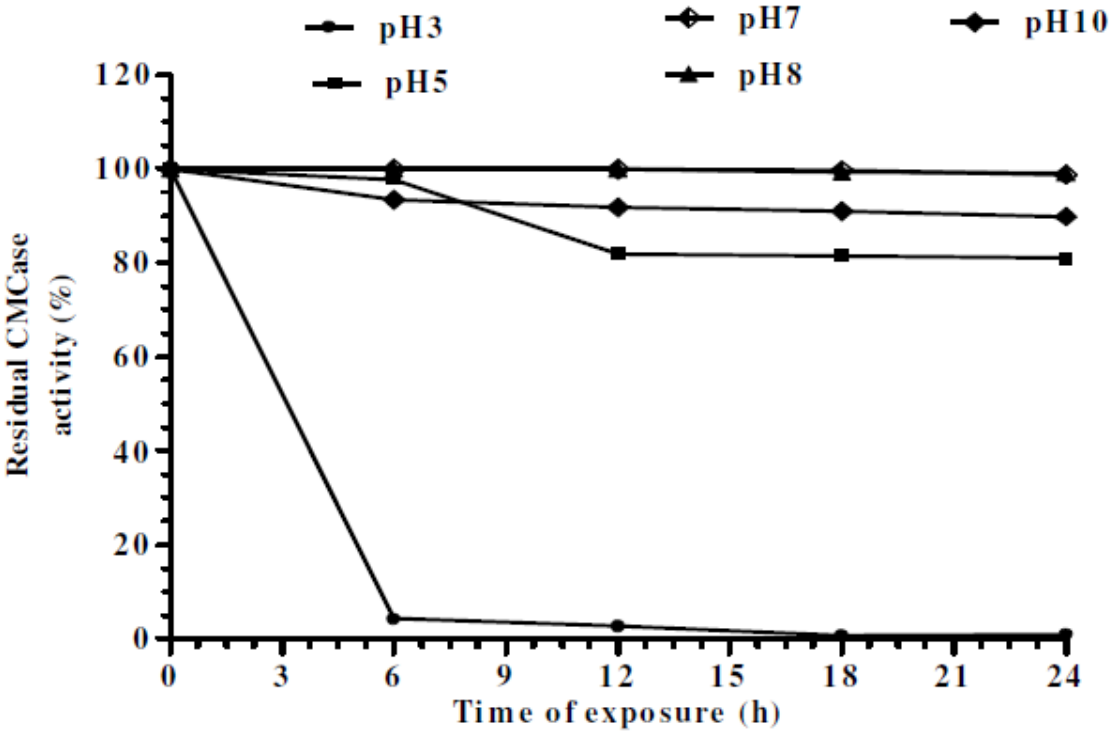

Figure 5F. pH stability of CMCase activity at different times.

relative activity for enzyme after each pH treatment was determined after adjusting the pH to the optimum one.

The enzyme was stable over a broad pH range from pH 7 to 10. The enzyme retained full activity at pH 7.0 to 8.0 for 6 h. The enzyme tolerated a higher pH; the enzyme retained 91.766% of the initial activity at pH 10, after incubation at 30°C for 12 h (Figure 5F). The enzyme tolerated a higher pH than the enzyme reported by George et al. (2001) who found that the CMCase from *Thermomonospora* retained up to 70% activity in the pH range 6 to 10 for 1 h. The ability to retain high activity at elevated pH is a potentially useful property. Alkaline enzymes, such as proteinases, lipases, and cellulases, have been used in the formulation of household detergents for the improvement of their efficiency. This allowed the reduction of the phosphate level in the conventional formulation of detergents, without decreasing their efficiency and with environmental importance (Dasilva et al., 1993).

HPLC analysis of free sugars released by enzymatic saccharification of rice straw

During the processing of the biomass, a portion of these polysaccharides were hydrolyzed and soluble sugars were released into the liquid fraction of the fermentation residues. As indicated in Figure 6, HPLC analysis identified the formation of glucose, cellobiose, mannose and xylose as saccharide byproducts. The formation of glucose and cellobiose suggests the sequential action of endoglucanase, exoglucanase and cellobiase since cellobiase activity would result in glucose formation, whereas the presence of mannose and xylose suggested the synergistic action between cellulases and xylanase in the degradation of pretreated rice straw (Lo et al., 2009).

Conclusion

Streptomyces sp. NEAE-D, an alkali-tolerant organism, is capable of successfully growing at high alkaline pH. The CMCase was stable over a broad pH range from pH 7 to 10. This reflects the future potential of the organism for successful and economic production of alkalistable CMCase. The relatively high alkali stability displayed by the CMCase is a valuable characteristic for its application in processes such as detergents industries. Furthermore, the organism successfully utilized crude carbon sources such as pre-treated rice straw and saw dust and produced much higher titre of CMCase than that reported on carboxymethyl cellulose. Using low-cost agro-industrial waste for the production of CMCase would help to reduce the cost of enzyme production and expand the application field of the CMCase. Characterization of the crude enzyme, as the optimum of pH and temperature and thermal stability, indicates the possibility of its biotechnological applications.

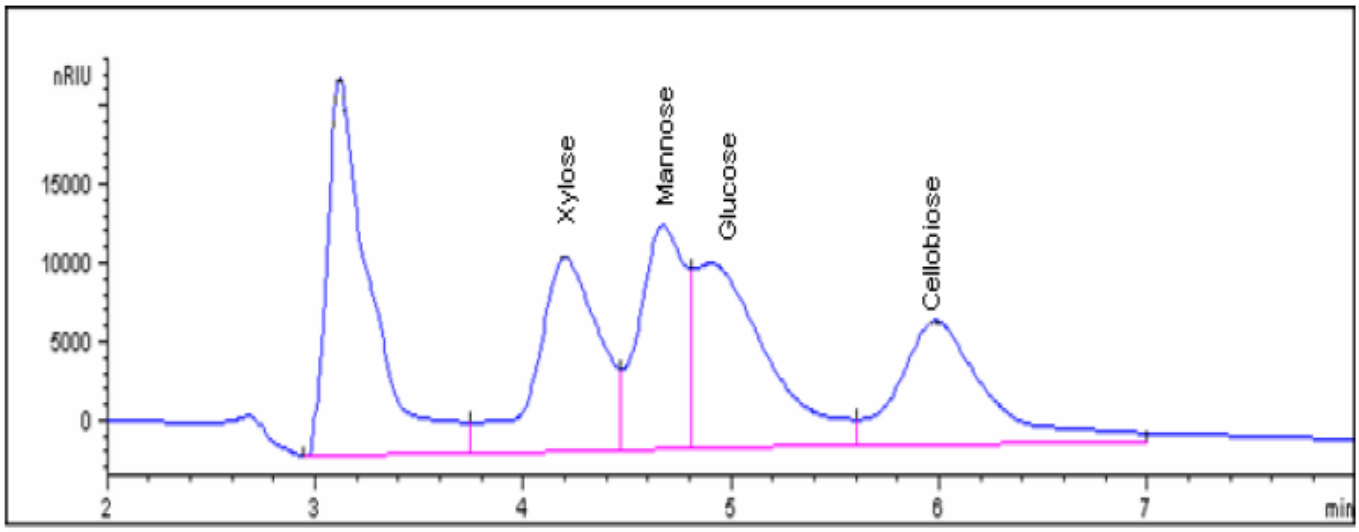

Figure 6. Chromatograph of the HPLC analysis of free sugars released by enzymatic saccharification of rice straw.

REFERENCES

Alam MZ, Manchur MA, Anwar MN (2004). Isolation, purification, characterization of cellulolytic enzymes produced by the isolate *Streptomyces omiyaensis*. Biol. Sci. 7: 1647-1653.

Apun K, Jong BC, Salleh MA (2000). Screening and isolation of a cellulolytic and amylolytic *Bacillus* from sago pith waste. J. Gen. Appl. Microbiol. 46: 263-267.

Ball AS, Godden B, Helvenstein P, Penninckx MJ, McCarthy AJ (1990). Lignocarbohydrate solubilization from straw by actinomycetes. Appl. Environ. Microbiol. 56: 3017-3022.

Bhat MK (2000). Cellulases and related enzymes in biotechnology. Biotech. Adv. 18: 355-383.

Chellapandi P, Himamhy M J (2008). Production of endoglucanse by the native strain of *Streptomyces* isolates in submerged fermentation. Braz. J. Microbiol. 39: 122-127

Dasilva R, Yim DK, Asquieri ER, Park YK (1993). Production of microbial alkaline cellulase and studies of their characteristics. Rev. Microbiol. 24: 269-274.

Dixon M, Webb EC (1971). Enzymes 2nd Edition,. Longman group Ltd. London. pp. 67-188.

Dosoretz CG, Chen HC, Grethlein HE (1990). Effect of environmental conditions on extracellular protease activity in lignolytic cultures of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 56: 395-400.

El-Naggar NE, Sherief AA, hamza SS (2011). Bioconversion process of rice straw by thermotolerant cellulolytics *Streptomyces viridiochromogenes* under solid-state fermentation condition for bioethanol production. Afrc.J. Biotechnol. 10(56): 11998-12011.

Galante YM, De Conti A, Monteverdi R (1998a). Application of *Trichoderma* enzymes in food and feed industries. In: Harman GF and Kubicek CP (Eds). *Trichoderma* and *Gliocladium*: Enzymes, biological control and commercial applications. Taylor and Francis, Vol. 2. London. pp. 327-342.

George SP, Ahmad A, Rao MB (2001). Studies on carboxymethyl cellulase produced by an alkalo-thermophilic actinomycete. Bioresour. Technol. 77: 171-175.

Gill PK, Sharma AD, Harchand RK, Singh P (2003). Effect of media supplements and culture conditions on inulinase production by an actinomycete strain. Bioresour. Technol. 87: 359-362.

Harchand RK, Singh S (1997). Characterization of cellulase complex of *Streptomyces albaduncus*. J. Basic. Microbiol. 37(2): 93-103.

Hopkins DW, Bibb MJ, Chater KF, Kieser C, Bruton CJ, Kiezer HM, Lydiate DJ, Smith CP, Ward JM, Schrempf H (1985). Genetic manipulation of *Streptomyces*. A laboratory manual. Norwich, UK: The John Innes Institute.

Hoshino E, Wada Y, Nishizawa K (1999). Improvements in the hygroscopic properties of cotton cellulose by treatment with an endotype cellulase from *Streptomyces* sp. KSM-26. J. Biosci. Bioeng. 88: 519-25.

Ishaque M, Kluepfel D (1980). Cellulase complex of a mesophilic *Streptomyces* strain. Can. J. Microbiol. 26(2): 183-189.

Jang HD, Chen KS (2003). Production and characterization of thermostable cellulases from *Streptomyces transformant* T3-1. W.J. Microbiol. Biotechnol. 19: 263-268.

Jaradat Z, Dawagreh A, Ababneh Q, Saadoun I (2008). Influence of culture conditions on cellulase production by *Streptomyces* sp. (strain J2). Jordan. J. Biol. Sci. 1: 141-146.

Kocher G, Kalra K, Banta G (2008). Optimization of cellulase production by submerged fermentation of rice straw by *Trichoderma harzianum* Rut-C 8230. The Internet J. Microbiol. Vol. 5, Num 2.

Kluepfel D, Shareck F, Mondou F, Morosoli R (1986). Characterization of cellulase and xylanase activities of *Streptomyces lividans*. Appl. Microbiol. Biotechnol. 24: 230-234.

Kotchoni OS, Shonukan OO, Gachomo WE (2003). *Bacillus pumillus*, BPCR16, a promising candidate for cellulase production under conditions of catabolic repression. Afr. J. Biotechnol. 2: 140-146.

Lacey J (1997). Actinomycetes in composts. Ann. Agric. Environ. Med. 4: 113-121.

Lima ALG, Nascimento RP, Bon S, Coelho RRR (2005). *Streptomyces drozdowiczii* cellulase production using agro-industrial by-products and its potential use in the detergent and textile industries. Enzyme Microb. Technol. 37: 272-277.

Lo YC, Saratale GD, Chen WM, Bai MD, Chang JS (2009). Isolation of cellulose-hydrolytic bacteria and applications of the cellulolytic enzymes for cellulosic biohydrogen production. Enzyme Microb. Technol. 44(6-7): 417- 425.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin Phenol reagent. Biol. Chem. 193: 265 - 275.

Mason MG, Ball AS, Brandon JR, Silkstone G, Nicholls P, Wilson MT (2001). Extracellular heme peroxidase in actinomycetes: a case of mistaken identity. Appl. Environ. Microbiol. 67: 4512-4519.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426- 428.

Muthezhilan R, Ashok R, Jayalakshmi S (2007). Production and optimization of thermostable alkaline xylanase by *Penicillium oxalicum* in solid state fermentation. Afr. J. Microbiol. 1: 20-28.

Ogel ZB, Yarangumeli K, Dundar H, Ifrij I (2001). Submerged cultivation of *Scytalidium thermophilum* on complex lignocellulosic biomass for endoglucanase production. Enzyme Microb. Technol. 28: 689-695.

Okeke BC, Paterson A (1992). Simultaneous production and induction of cellulolytic and xylanolytic enzymes in a *Streptomyces* sp. World J. Microbiol. Biotechnol. 8: 483-487.

Okeke BC, Obi SKC (1993). The production of cellulolytic and xylanolytic enzymes by an *Anthrographiae sp.* World J. Microbiol. Biotechnol. 9: 345-349.

Onsori H, Zamani MR, Motallebi M, Zarghami N (2005). Identification of over producer strain of endo-B-1, 4-glucanase in *Aspergillus* species: Characterization of crude carboxymethyl cellulase. Afr. J. Biotechnol. 4(1): 26-30.

Parry NJ, Beever DE, Owen E, Nerinckx W, Claeyssens M, Van Beeumen J (2002). Biochemical characterization and mode of action of a thermostable endoglucanase purified from *Thermoascus aurantiacus*. Arch. Biochem. Biophys. 404: 243-253

Schrempf H, Walter S (1995). The cellulolytic system of *Streptomyces retyiculi*. Int. J. Macromolecules, 15: 353-355.

Sherief AA, El-Naggar NE, Hamza SS (2010). Bioprocessing of lignocellulosic biomass for production of renewable bioethanol using thermotolerant *Aspergillus fumigates* under solid state fermentation conditions. Biotechnology, 9: 513-522.

Semedo L, Gomes R, Bon E, Soares R, Linhares L, Coelho R (2000). Endocellulase and exocellulase activities of two *Streptomyces* strains isolated from a forest soil. Appl. Biochem. Biotechnol. 84: 267-276

Stutzenberger FJ (1971). Cellulase production by *Thermomonospora curvata* isolated from municipal solid waste compost. Appl. Microbiol. 22: 147-152.

Theberge M, Lacaze P, Shareck F, Morosoli R, Kluepfel D (1992). Purification and characterization of an endoglucanase from *Streptomyces lividans* 66 and DNA sequence of the gene. Appl. Microbiol. 58: 815-820.

Tuncer M, Kuru A, Isikli M, Sahin N, Celenk FG (2004). Optimization of extra-cellular endoxylanase, endoglucanase and peroxidase production by *Streptomyctes* sp. F2621 isolated in Turkey. J. Appl. Microbiol. 97(4): 783-791.

Van Zyl WH (1985). A study of the cellulases produced by three mesophilic actinomycetes grown on bagasse as substrate. Biotechnol. Bioengi. 27(9): 1367-1373.

Wachinger G, Bronnemmeier K, Staundenbauer WL, Schrempf H (1989). Identification of mycelium-associated cellulase from *Streptomyces reticuli*. Appl. Environ. Microbiol. 55: 2653-2657.

Waksman SA (1959). Strain specificity and production of antibiotic substances. Characterization and classification of species within the *Streptomyces griseus* group. Proc. Natl. Acad. Sci. USA, 45: 1043-1047.

Xia L, Len P (1999). Cellulose production by solid-state fermentation on lignocellulosic waste from the xylose industry. Proc. Biochem. 34: 909-912.

Zhu L (2005). Fundamental study of structural features affecting enzymatic hydrolysis of lignocellulosic biomass. Doctorate Thesis, Texas A&M University. pp. 219-223.