Research Paper

Bioprocessing of some agro-industrial residues for endoglucanase production by the new subsp.; *Streptomyces albogriseolus* subsp. *cellulolyticus* strain NEAE-J

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Abstract

The use of low cost agro-industrial residues for the production of industrial enzymes is one of the ways to reduce significantly production costs. Cellulase producing actinomycetes were isolated from soil and decayed agricultural wastes. Among them, a potential culture, strain NEAE-J, was selected and identified on the basis of morphological, cultural, physiological and chemotaxonomic properties, together with 16S rDNA sequence. It is proposed that strain NEAE-J should be included in the species *Streptomyces albogriseolus* as a representative of a novel sub-species, *Streptomyces albogriseolus* subsp. *cellulolyticus* strain NEAE-J and sequencing product was deposited in the GenBank database under accession number JN229412. This organism was tested for its ability to produce endoglucanase and release reducing sugars from agro-industrial residues as substrates. Sugarcane bagasse was the most suitable substrate for endoglucanase production. Effects of process variables, namely incubation time, temperature, initial pH and nitrogen source on production of endoglucanase by submerged fermentation using *Streptomyces albogriseolus* subsp. *cellulolyticus* have been studied. Accordingly optimum conditions have been determined. Incubation temperature of 30 °C after 6 days, pH of 6.5, 1% sugarcane bagasse as carbon source and peptone as nitrogen source were found to be the optimum for endoglucanase production. Optimization of the process parameters resulted in about 2.6 fold increase in the endoglucanase activity. Therefore, *Streptomyces albogriseolus* subsp. *cellulolyticus* coud be potential microorganism for the intended application.

Key words: agro-industrial residues, endoglucanase production, *Streptomyces albogriseolus*, 16S rRNA, scanning electron microscope.

Introduction

In the recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues. However, the huge amounts of residual plant biomass considered as waste can potentially be converted into various different value added products including biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds and single cell products (Howard *et al.*, 2003; Ojumu *et al.*, 2003). Agro-industrial wastes have been used as carbon, hydrogen, and oxygen sources to produce ethanol, proteins, and microbial enzymes (Oliveira *et al.*, 2006). One of the largest cellulosic agro-industrial byproducts is sugarcane bagasse. It is a by-product of the sugar industry and is used by the sugar mills as fuel for boilers. There is an increasing trend towards more efficient utilization of agro-

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industrial wastes, including sugarcane bagasse and one of the significant applications is the production of enzymes. Although the economy of such processes is affected by the high cost of product, the utilization of cheaper and indigenous substrates has contributed somewhat to the economical recovery (Pandey *et al.*, 2000).

Endoglucanase (EC 3.2.1.4; endo-β- 1, 4 - glucanase) is among the industrially important hydrolytic enzymes, which one of great significance in present day biotechnology. It is found to have potential applications in different industries, like food processing (Galante *et al.*, 1998), animal feed production, pulp and paper industry for various purposes (Ogel *et al.*, 2001), and in detergent and textile industry (Kotchoni *et al.*, 2003) and also for the significant role in bioconversion of agricultural wastes into sugar and bioalcohols. Much research efforts have been focused on lowering the cost of enzymes (El-Naggar et al., 2014). The cost of carbon source plays major role in the economics of endoglucanase production. Hence, an approach to reduce the cost of endoglucanase production is the use of low cost lignocellulosic materials as substrates.

Actinomycetes comprise an extensive and diverse group of Gram-positive, aerobic, largely mycelial bacteria, which play an important ecological role in biodegradation. Many are commercially important, either in useful biological processes such as biodegradation and waste treatment, or in the pro-duction of bioactive compounds such as antibiotics and enzymes (Williams, 1985). *Streptomyces* species are the most industrially important actinomycetes, due to their capacity to produce numerous secondary metabolites (Berdy, 1995). However, soil isolates *Streptomyces lividens* (Kluepfel *et al.*, 1986), *Streptomyces* sp.T3-1(Hsu *et al.*, 2011), *S. albaduncus* (Harchand and Singh, 1997), S. *reticuli* (Schrempf and Walter, 1995) and *Streptomyces* sp. F2621 (Tuncer *et al.*, 2004) have already been reported to produce extracellular endoglucanase significantly in optimized culture media using cellulose powder as carbon source under shaking conditions.

The objectives of the present study were isolation of actinomycete strains, which have cellulolytic activities, selection and identification of a potential strain and also to maximize endoglucanase production by optimizing fermentation conditions using sugarcane bagasse as substrate in order to reduce the cost of production.

Materials and Methods

Collection of lignocelluloses

Different agricultural wastes like saw dust, rice husk, rice straw, wheat bran, oatmeal and sugarcane bagasse were collected from local fields of Dakahliyah governorate, Egypt, were oven-dried at 65 °C for two days to reduce the moisture content and then milled to achieve the size of less than 1 cm.

Microorganisms and cultural conditions

Streptomyces spp. used in this study were kindly provided by Dr. Noura El-Ahmady El-Naggar (Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technologial Applications, Alexandria, Egypt). Streptomyces spp. were isolated from various soil samples collected from different localities of Egypt (32 soil and decayed rice straw samples) and Saudi Arabia (3 soil samples). Actinomycetes from the soil had been isolated using standard dilution plate method procedure on Petri plates containing starch nitrate agar medium (Waksman, 1959) of the following composition (g/L): Starch, 20; KNO_3, 2; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $CaCO_3$, 3; $FeSO_4.7H_2O$, 0.01; agar, 20 and distilled water up to 1 L; then plates were incubated for a period of 7 days at 30 °C. Nystatin (50 μg/mL) was incorporated as an antifungal agent to minimize fungal contamination. *Streptomyces* isolates were purified and maintained as spore suspensions in 20% (v/v) glycerol at -20 °C (Hopkins *et al.*, 1985) for subsequent investigation.

Plate screening of endoglucanase

For plate screening, caboxymethylcellulose-agar (CMC-Agar) medium was used. This medium consists of (g/L): CMC, 10.0; $NaNO_3$, 1.2; KH_2PO_4, 3.0; K_2HPO_4, 6.0; $MgSO_47H_2O$, 0.2; $CaCl_2$, 0.05; $MnSO_47H_2O$, 0.01; $ZnSO_47H_2O$, 0.001; pH 7.0; agar, 20 and distilled water up to 1 L. Agar plates were seeded with spores of actinomycetes and incubated at 30 °C for 6 days, the cultures were then incubated further at 50 °C for 18 h to accelerate the action of extracellular cellulases and thus rapidly develop clearing zone around the cellulase-producing colonies (Montenecourt and Eveleigh, 1977). 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 15-20 min (Apun *et al.*, 2000), Unstained areas (clear zone of hydrolysis) indicate where the CMC has been broken down to β 1 → 4 glucans that contain seven or fewer glucose residues (Pragya *et al.*, 2012). 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.

Morphology and cultural characteristics

The morphology of the spore chain and the spore surface ornamentation of strain NEAE-J were examined on inorganic salt/starch agar (ISP medium 4) after 14 days at 30 °C. The gold-coated dehydrated specimen can be examined at different magnifications with Analytical Scanning Electron Microscope Jeol JSM-6360 LA operating at 20 Kv

at the Central Laboratory, City for Scientific Research and Technological Applications, Alexandria, Egypt. Aerial spore-mass colour, substrate mycelial pigmentation and the production of diffusible pigments were observed on tryptone -yeast extract agar (ISP medium 1), yeast extract-malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3), inorganic salt starch agar (ISP medium 4), glycerol- asparagine agar (ISP medium 5) peptone -yeast extract iron agar (ISP medium 6) and tyrosine agar (ISP medium 7) as described by Shirling and Gottlieb (1966), and on starch-ammonium sulphate agar; all plates were incubated at 30 °C for 14 days.

Physiological characteristics

Carbon source utilization was tested on plates of ISP basal medium 9 (Shirling and Gottlieb, 1966) supplemented with a final concentration of 1% of the tested carbon sources. The plates were incubated at 30 °C and read after 14 days. Melanoid pigment production was examined on peptone-yeast extract-iron agar (ISP medium 6) and on tyrosine agar (ISP medium 7) (Shirling and Gottlieb, 1966). Growth in the presence of sodium chloride was determined according to Tresner *et al.* (1968). Degradation of casein was tested following the method of Gordon *et al.* (1974) and reduction of nitrates to nitrites was examined according to Williams *et al.* (1983). Liquefaction of gelatin was evaluated by using the method of Waksman (1961). The ability to coagulate or to peptonize milk and hydrogen sulphide production was determined as described by Cowan and Steel (1974). Lecithinase activity was conducted on egg-yolk medium according to the method of Nitsch and Kützner (1969) and the capacity to decompose cellulose was tested following the method of Ariffin *et al.* (2006). The ability of strain to produce α-amylase (starch degradation) was determined by streaking onto starch nitrate medium plates containing 2% soluble starch and incubated at 30 °C for 7 days. After incubation, the plate is flooded with Gram's iodine solution and zone of clearance was observed (Mishra and Behera, 2008). The ability of the organism to inhibit the growth of four bacterial (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, or *Klebsiella* sp.), and five fungal strains (*Rhizoctonia solani*, *Alternaria solani*, *Bipolaris oryzae*, *Fusarium oxysporum*, *Fusarium solani*) was determined. Some additional tests can be considered to be useful in completing the description of a strain or species, even if they are not very significant or indicative on their own. The ability of strain NEAE -J to produce uricase (Azab *et al.*, 2005); asparaginase (Gulati *et al.*, 1997) and chitosanase (Choi *et al.*, 2004) were tested.

Chemotaxonomy

Sugars and diaminopimelic acid isomers were identified by thin-layer chromatography by the method described by Staneck and Roberts (1974).

16S rRNA sequencing

The preparation of genomic DNA of the strain was conducted in accordance with the methods described by Sambrook *et al.* (1989). The PCR amplification reaction was performed in a total volume of 100 μL, which contained 1 μL DNA, 10 μL of 250 mM deoxyribonucleotide 5'-triphosphate (dNTP's); 10 μL PCR buffer, 3.5 L 25 mM MgCl$_2$ and 0.5 μL Taq polymerase, 4 μL of 10 pmol (each) forward 16S rRNA primer 27f (5'-AGAGTTTGATCMTGCCTCAG-3') and reverse 16S rRNA primer 1492r (5'-TACGGYTACCTTGTTACGACTT-3') and water was added up to 100 μL. The PCR-Apparatus was programmed as follows: 5 min denaturation at 94 °C, followed by 35 amplification cycles of 1 min at 94 °C, 1 min of annealing at 55 °C, and 2 min of extension at 72 °C, followed by a 10 min final extension at 72 °C. The PCR reaction mixture was then analyzed via agarose gel electrophoresis, and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The purified PCR product of approximately 1400 bp was sequenced by using two primers, 518F; 5'-CCA GCA GCC GCG GTA ATA CG-3' and 800R; 5'-TAC CAG GGT ATC TAA TCC-3'. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing product was resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) and deposited in the GenBank database under accession number JN229412.

Sequence alignment and phylogenetic analysis

The nearly complete resultant 16S rRNA gene sequence (1006 nucleotides) of strain NEAE-J was aligned with the corresponding 16S rRNA sequences of the type strains of representative members of the genus *Streptomyces* retrieved from the GenBank, EMBL, DDBJ and PDB databases by using BLAST program (www.ncbi.nlm.nih. gov/blst) (Altschul *et al.*, 1997). The software package MEGA4 version 2.1 (Tamura *et al.*, 2007) was used for multiple alignment and phylogenetic analysis. The phylogenetic tree was constructed via the neighbor-joining algorithm (Saitou and Nei, 1987) based on the 16S rRNA gene sequences of strain NEAE-J and related organisms.

Fermentation conditions for endoglucanase production and saccharification

Submerged fermentation for enzyme production was carried out in Erlenmeyer flasks (250 mL) containing 50 mL of the production medium containing (g/L): sugarcane bagasse, 10.0; NaNO$_3$, 1.2; KH$_2$PO$_4$, 3.0; K$_2$HPO4, 6.0; MgSO47H$_2$O, 0.2; CaCl$_2$, 0.05; MnSO$_4$7H$_2$O, 0.01; ZnSO$_4$7H$_2$O, 0.001; yeast extract, 0.5 and distilled water up to 1L. The pH was adjusted to 7.0. The flasks were auto-

claved at 121 °C for 20 min, inoculated with three agar disks of 9 mm diameter taken from 7 days old stock culture (According to the method of Gill *et al.* (2003) and incubated at 30 °C for 6 days on a rotary shaker at 200 rpm. After incubation period, the cells were removed by centrifugation at 5000 rpm; the mycelium free supernatant was used as a crude enzyme for estimation of endoglucanase activity, the total protein and saccharification percentage.

For optimizing the fermentation conditions for both endoglucanase production and saccharification percentage, various parameters were studied for maximal enzyme production as follows: Different types of carbon sources for the induction of endoglucanase *i.e.*, cellobiose, cellulose powder, carboxymethyl cellulose, rice straw, wheat bran, sugarcane bagasse, saw dust, rice husk, oatmeal, xylan, sucrose and starch, which were separately added as a sole carbon source to the culture media at concentration of 1% (w/v). Sugarcane bagasse was then added to the basal salt media at several concentrations (0. 5 to 2%; w/v). Media were inoculated and incubated at 30 °C for 6 days with shaking at 200 rpm. The effect of incubation period was evaluated at 24 h interval. The effect of temperature on the production of endoglucanase was determined at various temperatures ranging from 20 to 50 °C at pH 6.5. The effect of pH-value on the production of endoglucanase was studied at various pH values ranging from 4.0 to 9.0. The effect of supplementation of nitrogen sources was examined using nitrogen sources like were ammonium chloride, ammonium phosphate, potassium nitrate, sodium nitrate, urea, yeast extract, peptone. Different nitrogen sources prepared at equivalent nitrogen bases were used separately as nitrogen source in production medium. Whereas, a control represented by production medium without additional nitrogen source was performed at the same time.

Endoglucanase assay

Endoglucanase activity in culture supernatant 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 culture supernatant 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, cooled down to room temperature. The O.D. of the samples was immediately measured at 540 nm. The determination was carried out in duplicate. Endoglucanase is expressed in terms of units. One unit of endoglucanase activity was defined as the amount of enzyme required to release 1 μmole reducing sugars per minute per mL under the assay conditions by using glucose as a standard.

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.

Saccharification

The percentage of saccharification was calculated by applying the equation of Alam *et al.* (2004):

$$\text{Saccharification }(\%) = \frac{\text{mg of reducing sugar / mL}}{\text{mg of substrate / mL}} \times 100$$

Results and Discussion

A total of one hundred forty one morphologically different actinomycete strains were recovered from 35 samples, which were collected from different localities in Egypt and Saudi Arabia. Actinomycetes strains were examined for cellulolytic activities on CMC- containing agar plates. Upon initial screening, it appeared that most of the isolates (77.3%) were able to develop clear surrounding zone indicating cellulolytic activities and cellulase production while only 22.7% of the isolates were unable to produce this enzyme. Cellulase producing isolates were categorized into 4 groups according to the width of inhibition zones; very strong (> 30 mm), strong (21-30 mm), moderate (11-20 mm), weak (1-10 mm), the four groups were represented by 1.42, 44.68, 29.79 and 1.42% activity, respectively (Figure 1a). Strain NEAE-J showed large clear zone diameter around its colonies (Figure 1b). The cellulolytic activity of the selected strain was confirmed under submerged fermentation indicating the highest cellulose degradation.

Cultural and morphological characteristics of the isolate NEAE-J

The colonial morphology of strain NEAE-J was consistent with its assignment to the genus *Streptomyces* (Williams *et al.*, 1989). It formed an extensively branched substrate mycelium and aerial hyphae. The aerial spore mass was grey colored on several standard media (Figure 2). The reverse sides of colonies are faint-brown on most agar media (Table 1). The color of the substrate mycelium is not pH sensitive and faint brown diffusible pigments were produced on inorganic salt-starch agar medium (ISP medium 4).

The organism formed a highly branched substrate mycelium and aerial hyphae which differentiated into long rectiflexibiles spore chains carrying more than 50, smooth-surfaced spores (0.62 x 0.881 μm in diameter) as shown by the scanning electron micrograph (Figure 3).

Strain NEAE -J can be differentiated from *S. albogriseolus* and other closest phylogenetic neighbours by many features (Table 2) in that it produced a gray aerial mycelium, brown substrate mycelial pigment and produce faint brown diffusible pigment on ISP2 medium while *S.*
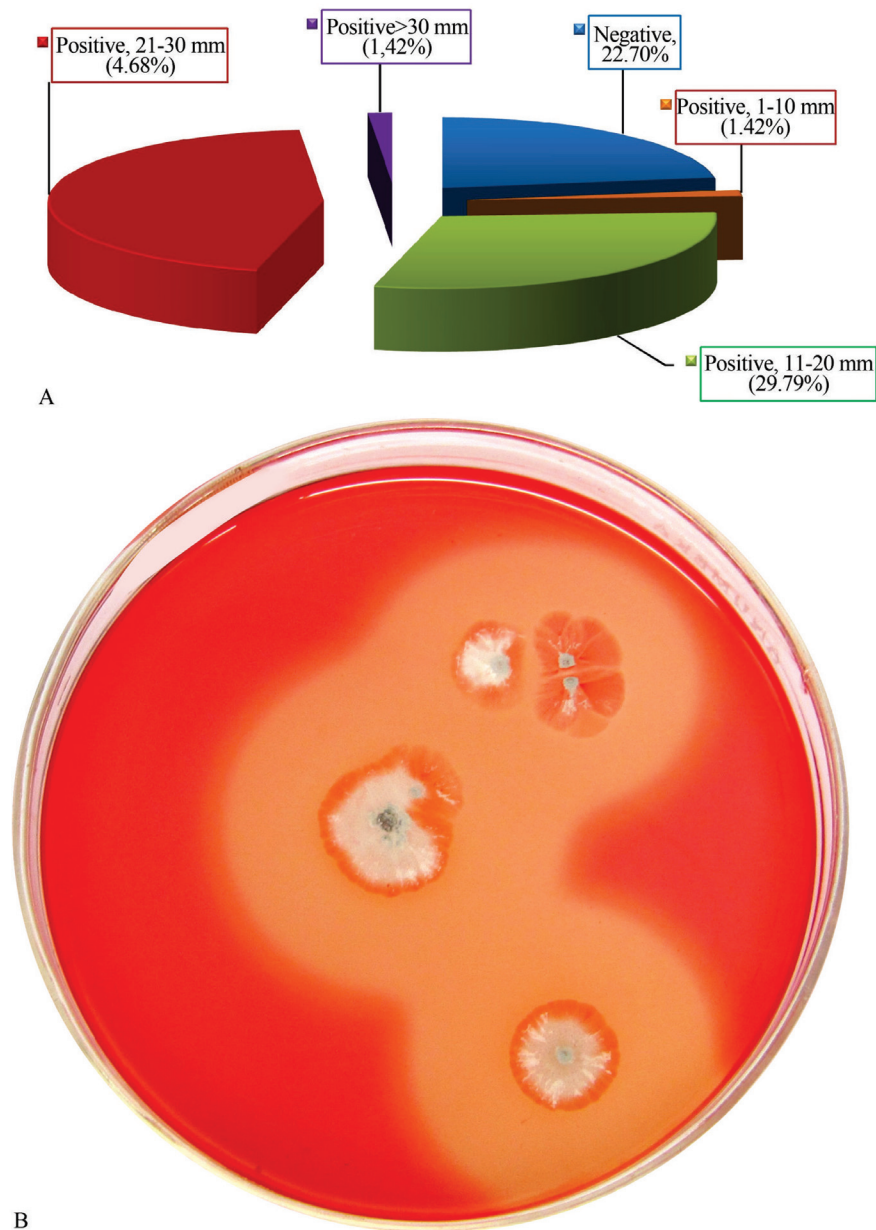
Figure 1 - A- Screening of actinomycetes isolates for the degradation of caboxymethylcellulose on CMC-agar (percentage inside the parenthesis indicates the frequency of actinomycetes isolates within specific category in relation to the total isolates). B- Cellulolytic activity of *Streptomyces* sp. NEAE -J on cellulose agar indicated by clearing zone surrounding the colony.

albogriseolus produced gray aerial mycelium, yellow/ brown substrate mycelium pigment and did not produce a diffusible pigment on ISP2 medium (Benedict *et al.*, 1954); *S. carnosus* produced a grey aerial mycelium , grayish yellow to orange yellow or brown substrate mycelial and pale pink or yellow diffusible pigment in oatmeal agar and glycerol-asparagine agar (Waksman and Lechevalier, 1953); *S. litmocidini* produced a grey aerial mycelium, colorless or grayed yellow substrate mycelial and violet, blue or red diffusible pigment (Pridham *et al.*, 1958)and *S. pactum* produced a beige aerial mycelium, grey substrate mycelial and beige diffusible pigment (Bhuyan *et al.*, 1962).

Physiological characteristics of the isolate NEAE-J

The physiological and biochemical reactions of strain NEAE-J are shown in Table 2. Melanoid pigments not formed neither in peptone-yeast extract iron agar (ISP medium 6) or tyrosine agar (ISP medium 7). As the sole carbon source, *Streptomyces* NEAE-J utilizes D-glucose, D-xylose, D-fructose, D-maltose, D-mannose, rhamnose, raffinose, L-arabinose and cellulose for growth. Ribose and gluconic acid are not utilized. Some growth occurs with galactose and sucrose as the carbon source. The strain degrades casein, gelatin, cellulose and starch. Lecithinase ac-

Figure 2 - Color of the aerial mycelium of *Streptomyces* strain grown on starch -nitrate agar medium for 14 days of incubation at 30 °C.

tivity, nitrate reduction and hydrogen sulphide production were negative. Coaggulation of milk was doubtful. Protease, cellulase, amylase, uricase and chitosanase were produced L-asparaginase. The optimal growth temperature of strain NEAE-J was 30 °C and optimal pH was 7.0.

Compared with *S. albogriseolus* (Benedict *et al.*, 1954), strain NEAE -J differed in a number of characteristics, including antimicrobial activities and sucrose utilization. Compared with *S. carnosus* (Waksman and Lechevalier, 1953), differences were observed in reduction of nitrates to nitrite, utilization of D (+) xylose, sucrose and L-arabinose, coaggulation and peptonization of milk. Strain NEAE -J differed from *S. litmocidini* in some characteristics, including sensitivity of diffusible pigment to pH, melanin production and utilization of D (-) fructose, D (+) xylose sucrose and raffinose (Pridham *et al.*, 1958). *S. pactum* also showed some differences in relation to strain NEAE -J, including the antimicrobial activities and utilization of D (-) fructose, D (+) xylose, D (+) mannose, sucrose, cellulose, rhamnose, raffinose and L-arabinose (Bhuyan *et al.*, 1962).
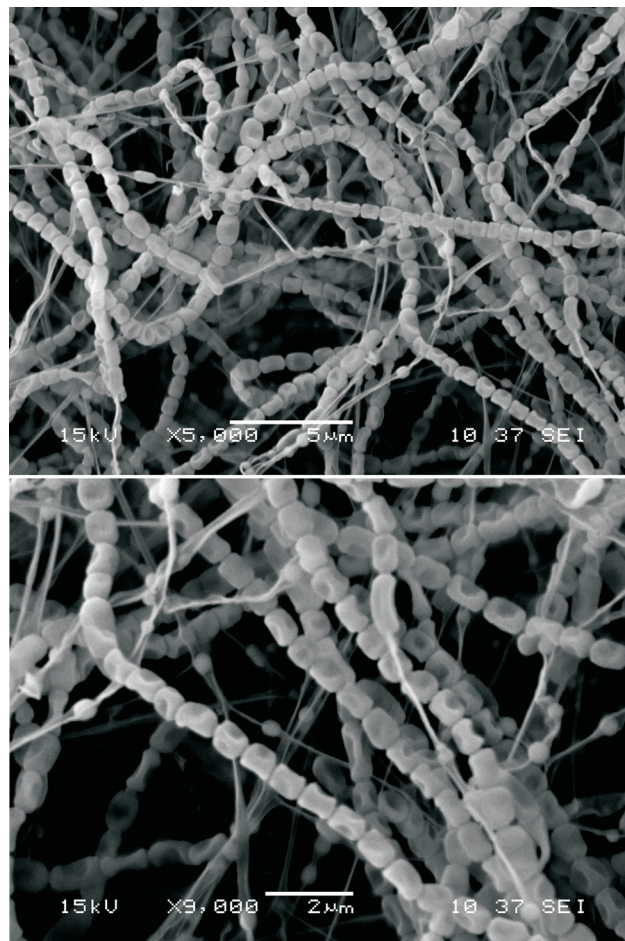
Figure 3 - Scanning electron micrograph showing the spore-chain morphology and spore-surface ornamentation of strain NEAE-J grown on starch nitrate agar medium for 14 days at 30 °C at magnification of 5000 X and 9000 X.

Chemotaxonomy of the isolate NEAE-J

Chemotaxonomic tests showed that the cell wall contained LL-diaminopimelic acid in whole-organism hydrolysates, indicating that it was of cell-wall type I (Lechevalier and Lechevalier, 1970) but no characteristic sugars were found in the strain.

Table 1 - Culture properties of the *Streptomyces* isolate NEAE-J.

Medium	Aerial mycelium	Substrate mycelium	Diffusible pigment	Growth
ISP 2 medium (Yeast extract -malt extract agar)	Gray	Faint brown	Non-pigmented	Excellent
ISP 3 medium (Oatmeal agar)	Whitish gray	Faint brown	Non-pigmented	Excellent
ISP 4 medium (Inorganic salt-starch agar)	Gray	Faint brown	Faint brown	Excellent
ISP 5 medium (Glycerol asparagines agar)	Faint growth with white margins	Faint yellow (Not distinctive)	Non-pigmented	Weak
ISP 6 medium (Peptone-yeast extract iron agar)	No-sporulation	No-sporulation	Non-pigmented	Weak
ISP 7 medium (Tyrosine agar)	Faint gray	Brown	Non-pigmented	Excellent
Starch ammonium sulphate	Gray	Faint yellow (Not distinctive)	Non-pigmented	Excellent

Table 2 - Phenotypic properties that separate strain *Streptomyces* NEAE-J from related *Streptomyces* species. *S. albogriseolus* (Benedict *et al.*, 1954), *S. carnosus* (Waksman and Lechevalier, 1953), *S. litmocidini* (Pridham *et al.*, 1958). *S. pactum* (Bhuyan *et al.*, 1962).

Characteristic	*Streptomyces* NEAE-J[1]	*S. albogriseolus*	*S. carnosus*	*S. litmocidini*	*S. pactum*
Aerial mycelium on ISP medium 2	Gray	Gray	Gray	Gray	Beige
Substrate mycelium on ISP medium 2	brown	yellow/ brown	Grayish yellow to orange yellow or brown	Colorless or grayed yellow	grey
Production of diffusible pigment	Faint brown	-	Pale pink or yellow in oatmeal agar and glycerol-asparagine agar	Violet, blue or red	beige
Spore chain morphology	RF	RF	RA or Spirales	RF or RA	RA
Number of spores /chain			10-50	10-50	
Spore surface	Smooth	Smooth	Smooth	Smooth	Smooth
Spore shape	Elongated		Cylindrical	Cylindrical	
Sensitivity of diffusible pigment to pH	-		-	+[2]	
Melanin production on tyrosine agar (ISP medium 7)	-		-	-	-
Melanin production on peptone-yeast extract iron agar (ISP medium 6)	-		-	+	-
Degradation of					
Casein	+				
Gelatin	+	+	liquefaction rapid		
Starch	+				
Max NaCl tolerance (+, w/v)	5%	5%			
Growth on sole carbon source (1+, w/v)					
D(-) Fructose	+	+		±	-
D(+) Xylose	+	+	±	±	-
D(+) Glucose	+	+	+	+	+
D(+) Mannose	+	+			-
Sucrose	±	+	±	±	-
D(+) Galactose	±				
Cellulose	+	+			-
Rhamnose	+	+	+		-
Raffinose	+	+		±	-
L-arabinose	+	+	±	+	-
Ribose	-				
Gluconic acid	-				
Reduction of nitrates to nitrite	-		+		
Lecithinase activity	-				
H₂S production	-	-			-
Coaggulation of milk	±		+		
Peptonization of milk	±		+		
Antimicrobial activities	-	+[3]			+[4]

[1]Protease, cellulase, amylase, L-asparaginase, uricase and chitosanase of *Streptomyces* NEAE-J were produced. The optimal growth temperature was 30 °C and optimal pH was 7.0.
[2]Reverse color is changed from violet to blue by addition of 0.05 N NaOH and from violet to red with 0.05N HC1.
[3]Produce Amphomycin and Cephamycin antibiotics that active against gram positive bacteria. Produce Vineomacin that has antibacterial and antitumor activities.
[4]Produce Pactamycin, antitumor antibiotic and others.
Abbreviations: RF, Rectiflexibiles; RA, Retinaculiaperti; S, Spirales; +, Positive; -, Negative; ± , Doubtful; Blank cells, no data available.

Molecular phylogeny of the isolate NEAE-J

The nearly complete 16S rRNA gene sequence (1006 nucleotides) for the strain NEAE-J was aligned with other 16S rRNA sequences of representative *Streptomyces* species retrieved from the GenBank, EMBL and DDBJ databases by using BLAST searches (Altschul *et al.*, 1997). The phylogenetic tree (Figure 4) based on the 16S rRNA gene sequences of strain NEAE-J and the most closely related type strains of the genus *Streptomyces* was constructed according to the neighbour-joining method of Saitou and Nei (1987) with MEGA4 (Tamura *et al.*, 2007). The phylogenetic analysis indicated that strain NEAE-J formed a distinct branch with two other *Streptomyces* species, *Streptomyces pactum* strain 173848 (GenBank/ EMBL/DDBJ accession no. EU593609.1) (99.0% sequence similarity) and *Streptomyces albogriseolus* strain HBUM174069 (GenBank/EMBL/DDBJ accession no. FJ486338.1) (99.0% sequence similarity). The comparative study between strain NEAE-J and its closest phylogenetic neighbours, however, revealed significant differences from those *Streptomyces* species as summarized in Table 2.

From the taxonomic features, the cellulolytic strain NEAE-J matches with *Streptomyces albogriseolus* in most morphological, physiological and biochemical characters and differed in some characteristics, including antimicrobial activities, production of diffusible pigment and utilization of sucrose. Therefore, it is proposed that strain NEAE-J should be included in the genus *Streptomyces albogriseolus* as a representative of a novel sub-species, *Streptomyces albogriseolus* subsp. *cellulolyticus* stain NEAE-J.

Description of *Streptomyces albogriseolus* subsp. *cellulolyticus*. nov.

Streptomyces albogriseolus subsp. *cellulolyticus* (*cellulolyticus* pertaining to cel.lu.lo.1y'ti.cus. M. L. n. *cellulosum,* cellulose; Gr. adj. *lyticus,* dissolving; M. L. adj. *cellulolyticus,* decomposing cellulose).

Aerobic, Gram-positive mesophilic actinomycete which forms an extensively branched substrate mycelium and aerial hyphae that differentiate into long rectiflexibiles spore chains with more than 50 smooth-surfaced spores per chain. Some unicellular spherical bodies are produced separately in some mycelial filament. It developed grey coloured aerial mycelium and faint brown substrate mycelium on yeast extract -malt extract agar. The colour of the substrate mycelium was not sensitive to changes in pH. Verticils are not present. The mycelium does not fragment. A diffusible faint brown pigment is produced in inorganic salt-starch agar. Melanin pigments are not produced on peptone/yeast extract/ iron or tyrosine agar. Additional cultural characteristics on various agar media are given in Table 1. Growth occurs between 20 and 40 °C and between pH 4.0 and 10.0. The optimal growth temperature was 30 °C and optimal pH was 7.0. The cell wall peptidoglycan contains LL-diaminopimelic acid (chemotype I cell wall). Strain NEAE-J grew well on yeast extract - malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3), inorganic salt-starch agar (ISP medium 4), tyrosine agar (ISP medium 7) and starch-ammonium sulphate agar medium. It exhibited weak growth on glycerol-asparagine agar (ISP medium 5) and peptone-yeast extract iron agar (ISP medium 6). Gelatin liquifications, growth on cellulose and starch hydrolysis (Figure 5) were positive. Lecithinase activity, nitrate reduction, melanin production, and hydrogen sulphide production were negative. Coaggulation of milk was doubtful. Protease, cellulase, amylase, uricase and chitosanase are produced L-asparaginase.

D-glucose, D-xylose, D-fructose, D-maltose, D-mannose, rhamnose, raffinose, L-arabinose and cellulose were utilized but not ribose and gluconic acid. Galactose and sucrose were doubtful. It did not exhibit any antimicrobial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp., *Rhizoctonia solani*, *Alternaria solani*, *Bipolaris oryzae*, *Fusarium oxysporum* or *Fusarium solani*.
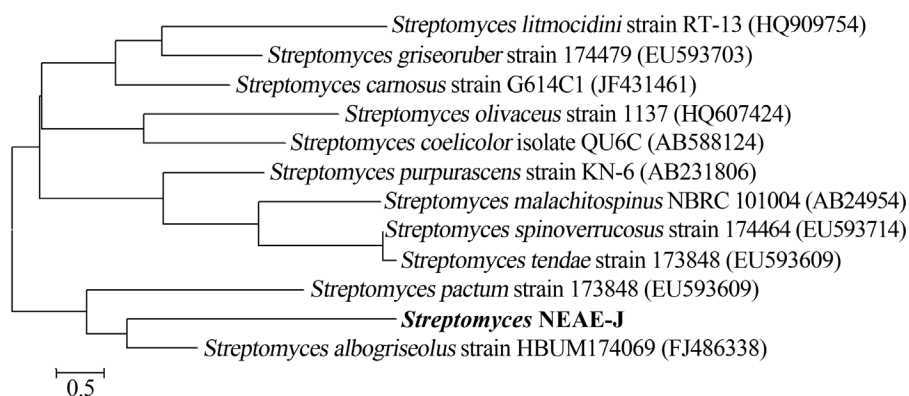
Figure 4 - Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain NEAE-J and related species of the genus *Streptomyces*. GenBank sequence accession numbers are indicated in parentheses after the strain names.

Figure 5 - Plate assay showing zone of hydrolysis of starch by strain NEAE 102. All the starch in the medium around the microbe has been hydrolyzed by extracellular amylases.

Endoglucanase production by *S. albogriseolus* subsp. *cellulolyticus* using different agro-industrial residues as carbon source

A comparative study was conducted to determine the suitability of common agro-industrial residues as well as some others carbon sources for endoglucanase production by *Streptomyces albogriseolus* subsp. *cellulolyticus* with that of control in which carboxymethyl cellulose is amended instead of agriculture by-products (Table 3a). Obtained data clearly show that endoglucanase was produced on the different sources used, but some of these sources reduced the enzyme biosynthesis, while other induced it greatly. Among these, xylan, sucrose, starch, inulin, oat-

meal and cellobiose had no significant stimulating effect on endoglucanase production by the tested strain while sugarcane bagasse, cellulose powder, rice straw, saw dust, wheat bran and rice husk significantly enhanced endoglucanase production as compared to that on pure CMC. This means that, this enzyme is constitutive in their nature and induced with its substrates. However, the maximum endoglucanase yield was achieved by sugarcane bagasse (41.54 U/mL) while the minimum production was recorded in presence of sucrose as carbon source (14.07 U/mL). Chellapandi and Himamhy (2008) showed that starch and maltose repressed endoglucanase production by *Streptomyces* sp. BRC1 and BRC2 respectively while glucose and cellobiose showed as good inducers to form endoglucanase by these isolates. El-Naggar *et al.* (2011) applied actinobacteria on rice straw composting and found their efficiency in rice straw decomposition. Production of this enzyme by a strain of *Streptomyces* sp. HM29 occurred with higher yields when the strain was grown on bagasse in comparison to rice straw, rye straw or corncobs (Modi *et al.*, 1994).

There were evidences for the efficiency of the biodegradation of the different carbon sources by the isolated strain. The evidences are the estimated amounts of reducing sugars and the saccharification, occurred on the different substrates, which reached, to the maximum in the case of sugarcane bagasses. The positive significant correlation co-efficient between enzyme production and saccharification ($r = 0.999$ at $p \leq 0.01$) confirmed the strong action of the endoglucanase. The specific activity of endoglucanase varied on different substrate, this may be back to the different growth patterns of *Streptomyces* strain as a response to various nature of the different carbon sources, CMC showed the highest value (26.51 U/mg protein) in this respect. The specific activity of endoglucanase, resulted from *S. albogriseolus* subsp. *cellulolyticus* grown on different carbon sources did not show specific trend. This may be back

Table 3a - Endoglucanase production and biodegradation of different carbon sources by *S. albogriseolus* subsp. *cellulolyticus*.

Carbon Source	Endoglucanase activity (U/mL)	Specific activity (U/mg protein)	Reducing sugars (mg/mL)	Saccharification (%)
CMC (control)	30.09	26.51	1.083	10.83
Cellulose	34.27	19.73	1.233	12.33
Cellobiose	18.45	10.72	0.664	6.64
Rice husk	30.59	14.77	1.101	11.01
Rice straw	33.25	20.52	1.196	11.96
Saw dust	32.67	13.25	1.176	11.76
Sugarcane bagasse	41.54	17.23	1.495	14.95
Wheat bran	31.86	18.29	1.146	11.46
Oatmeal	28.13	15.93	1.012	10.12
Sucrose	14.07	3.08	0.506	5.06
Starch	19.41	13.84	0.698	6.98
Xylan	24.28	16.76	0.874	8.74

to the different composition of carbon source which led to various growth natures of *S. albogriseolus* subsp. *cellulolyticus*, resulting different types of protein in the growth media. Therefore, no much dependency could be relying on such parameter. Accordingly, different concentrations of sugarcane bagasse (0.5, 1.0, 1.5 and 2%; w/v) were added to the cultivation medium. The maximum endoglucanase yield was obtained at 1.0% of sugarcane bagasse (Table 3b). The results of sugarcane bagasse follow the nature curve in which the peak of saccharification and enzyme production was at 1% with positive correlation (r = 0.999 at p ≤ 0.01), confirming the data of Table 3a.

The carbon source has been estimated as a major cost factor in enzyme production. Industrial application of endoglucanase, however, would only be feasible if the carbon sources were available in large quantities at competitive price. A reduction in the production cost can be achieved by the usage of inexpensive crude raw materials of agricultural origin, which are cheap, easily available, often abundant, alternative, for large-scale fermentation.

Effect of incubation period on endoglucanase production

It is observed from the results in the Figure 6 that the endoglucanase production gradually raised and reached its maximum activities (57.69 U/mL) after 6 days incubation,
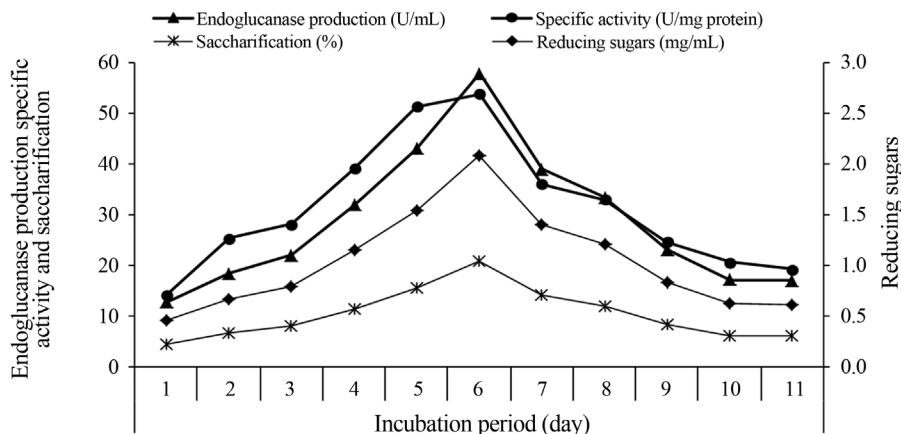
then gradually decreased. Additionally, all the tested parameters (specific activity, reducing sugars and saccharification) follow the same trend. This decline after the maximum peak of enzyme secretion could be attributed to catabolic repression by glucose, since the maximum amount of glucose was accumulated at the 6th day. Cellulase production is growth related, so, depletion of carbon and nitrogen sources causes starvation and hence the microorganism may not grow as reported by Dosoretz *et al.* (1990). The depression in cellulase activity may also be due to cumulative effect of cellobiose, a dimer of glucose which is known to inhibit endogluconase activity (Ojumu *et al.*, 2003).

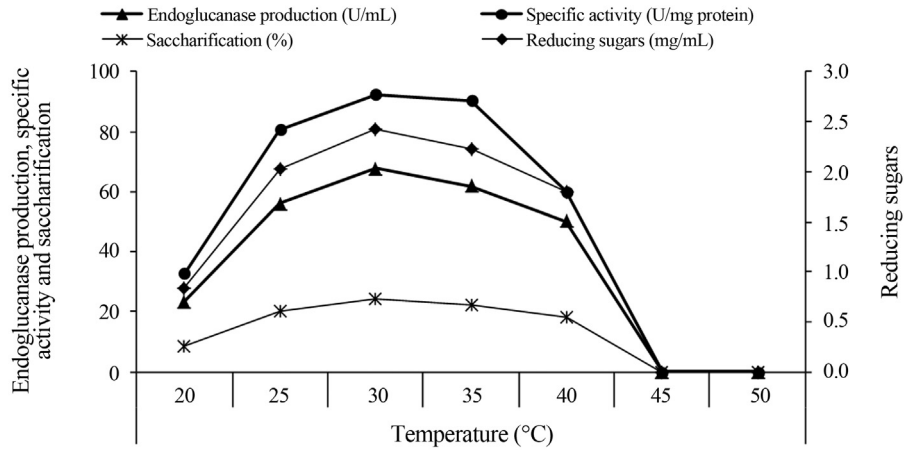## Effect of temperature on endoglucanase production

As shown in Figure 7, the enzyme showed a good production between 25 to 40 °C with maximum endoglucanase production (67.61 U/mL), reducing sugars (2.43 mg/mL) and saccharification (24.33 mg/mL) at 30 °C. Out this temperature, the enzyme production gradually reduced and the decrease is probably due to enzyme denaturation and conformation change, as enzymes are proteins. Our results are in accordance with two previous reports on endoglucanase by *Streptomyces* sp. F2621 (Tuncer *et al.*, 2004) and *S. albogriseous* (Van Zyl, 1985) which exhibited maximum endoglucanase activity at 26-30 °C. On the other hand, the results reported by El-Naggar and Abdelwahed (2012)

Table 3b - Effect of different sugarcane bagasse concentration on endoglucanase production, reducing sugars level and saccharification by *S. albogriseolus* subsp. *cellulolyticus*.

Carbon source concentration (%)	Endoglucanase activity (U/mL)	Specific activity (U/mg protein)	Reducing sugars (mg/mL)	Saccharification (%)
0.5	30.86	16.05	1.11	11.10
1	43.50	19.70	1.57	15.65
1.5	41.45	18.31	1.49	14.92
2	34.30	13.09	1.23	12.34

Figure 6 - Effect of incubation periods on endoglucanase production, reducing sugars level and saccharification of sugarcane bagasse by *S. albogriseolus* subsp. *cellulolyticus*.

Figure 7 - Effect of temperature on endoglucanase production, reducing sugars level and saccharification of sugarcane bagasse by *S. albogriseolus* subsp. *cellulolyticus*.

showed that the optimum temperature for endoglucanase from a strain of *Streptomyces* sp. strain NEAE-D was 35 °C.
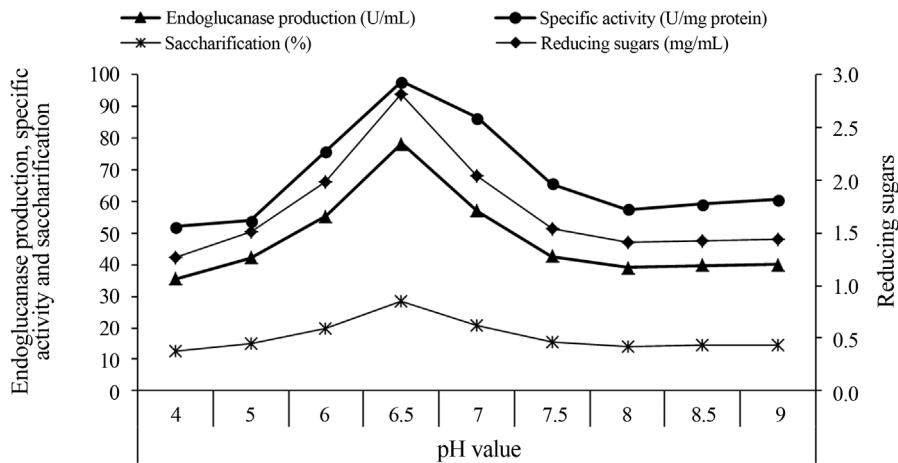
Effect of pH on endoglucanase production

The effect of cultural pH on endoglucanase production by *S. albogriseolus* subsp. *cellulolyticus* was examined at various values ranging from 4.0 to 9.0 as shown in Figure 8. The enzyme shows high activity (78.26 U/mL), reducing sugars (2.82 mg/mL) and saccharification (28.16 mg/mL) at pH 6.5. The enzyme production reduced by 49.91% at pH 8. Similarly, *Streptomyces omiyaensis* (Alam *et al.*, 2004) and *S. viridochromogenes* (El-Naggar *et al.*, 2011) recorded high cellulase activity at pH 6.5. *Streptomyces* sp. F2621 (Tuncer *et al.*, 2004) and *S. albogriseous* (Van Zyl, 1985) also exhibited maximum endoglucanase activity at initial pH of 6.5-7.0. On the other hand, the results appeared to contradict with these reported

by Theberge *et al.* (1992) in which, the optimum pH for endoglucanase from a strain of *Streptomyces lividans* was 5.5. Sherief *et al.* (2010) showed that the optimum pH for maximum production of CMCase from a strain of *Aspergillus fumigatus* was 5.5.

Influence of different nitrogen sources on endoglucanase production

In an attempt to maintain low fermentation costs during endoglucanase production, relatively inexpensive organic nitrogen sources (yeast extract, peptone and casein) and inorganic nitrogen sources (ammonium chloride, ammonium phosphate, potassium nitrate, sodium nitrate and urea) were used (Table 4). Among the nitrogen sources tested, peptone proved to be the best nitrogen source for enzyme production (107.71 U/mL) followed by yeast extract (94.82 U/mL). Peptone moreover, recorded the highest values of both reducing sugars and saccharification, confirm-

Figure 8 - Effect of pH on endoglucanase production, reducing sugars level and saccharification of sugarcane bagasse by *S. albogriseolus* subsp. *cellulolyticus*.

Table 4 - Effect of different nitrogen sources on endoglucanase production, reducing sugars level and saccharification of sugarcane bagasse by *S. albogriseolus* subsp. *cellulolyticus*.

Nitrogen Source	Endoglucanase activity (U/mL)	Specific activity (U/mg protein)	Reducing sugars (mg/mL)	Saccharification (%)
Control	37.02	141.28	1.33	13.32
NaNO$_3$ + Yeast extract	84.98	75.54	3.06	30.58
NaNO$_3$	66.39	66.86	2.39	23.89
KNO$_3$	79.34	84.22	2.85	28.55
Urea	77.41	160.63	2.79	27.85
Peptone	107.71	98.26	3.88	38.76
Casein	65.46	54.83	2.36	23.56
NH$_4$Cl	70.17	125.62	2.52	25.25
Yeast extract	94.82	96.94	3.41	34.12
NH$_4$H$_2$PO$_4$	59.65	98.19	2.15	21.47

ing the good growth and biodegradation of sugarcane bagasse by *S. albogriseolus* subsp. *cellulolyticus* on peptone as the sole nitrogen source. With respect to specific activity of endoglucanase, no clear trend could be detected, this may be back to the variation among nitrogen sources in their composition, where the growth nature of *S. albogriseolus* subsp. *Cellulolyticus* varied, as a result, the secreted protein varied in quantity and quality, leading to various specific activity, so no much dependency could be relying on such parameter. Our results are in accordance with the work of Enari *et al.* (1977) who reported that good cellulase production could be obtained with the organic nitrogen sources such as yeast extract and peptone. Yeast extract suited for enhanced production of endoglucanase by *Streptomyces* sp. BRC2 (Chellapandi and Himanshu, 2008). Yeast extract for *Streptomyces* sp. F2621 (Tuncer *et al.*, 2004) is reported as potential nitrogen source for endoglucanase production. Peptone was found to be the best nitrogen source for exoglucanase production (Mukherjee *et al.*, 2011).

Conclusion

Among different agro-industrial residues that used for endoglucanase production from *Streptomyces albogriseolus* subsp. *cellulolyticus*, the enzyme yield obtained from sugarcane bagasse is comparatively higher to that obtained from carboxymethyl cellulose. Therefore, it was thought that agro-industrial residues could be considered to be an alternative substrate for endoglucanase production by the novel subsp.; *S. albogriseolus* subsp. *cellulolyticus*. Besides that, it would be benefit for the economic endoglucanase production and alternative method to convert agricultural wastes into useful products.

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