Research Article

Optimization of the Production of a β-1, 4-endoglucanase from a Newly Isolated *Bacillus* sp.RL1 by Medium Optimization and Analysis Different Growth Parameters

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Abstract: The aim of this study was for the production of extracellular endoglucanase by a novel new strain of *Bacillus* sp. RL1 isolated from soil was improved by medium optimization. A low cost source of β-1, 4-Endoglucanase (EG) with robust activity is of potential commercial value. Here, we identified a soil bacterial strain, *Bacillus* sp. RL1, as a potential source for EG with high temperature tolerance. The culture parameters, such as duration of incubation, incubation temperature, pH, carbon and nitrogen sources and agitation speed and additives, were optimized for enhancing EG yield. The optimal level of each parameter for maximum EG production by *Bacillus* sp. RL1 was determined. Results showed that the EG production was higher 4-folds with pineapple containing production medium, beef extract, shaking 200 rpm and $CoCL_2$ and the incubation temperature, time course and pH were 35°C, 3rd day and 7.0 respectively, coparasion with basil medium (CMC). Thermal stability of EG was approximately 85.5% at 90°C for 30 min. The enzyme maintained stability over a wide range of pH from 4-10. In addition optimized medium containing agricultural wastes pineapple combined with best two nitrogen sources (organic and inorganic nitrogen sources) showed significant activation on EG, FPase, glucosidase and xylanase were 18-folds, 6.5 –folds, 14- folds and 13- folds, respectively.

Keywords: *Bacillus* sp. RL1, endoglucanase, new strain, optimization of enzyme production, pineapple

INTRODUCTION

Lignocellulosic biomass, an abundant and renewable carbon source, has the potential to replace starch as a feedstock for the production of fuel ethanol (Lee, 1997; Bischoff *et al*., 2007). The most common forms of carbon found in lignocellulosic biomass are cellulose, a bio-polymer of glucose and hemicellulose, a heterogeneous bio-polymer of hexose and pentose sugars including glucose, galactose, xylose and arabinose. The lack of efficient methods that degrade the lignocellulosic biomass to fermentable sugars poses a significant technical barrier to the widespread commercial use of this commodity for biofuel production.

Cellulolytic activity is a multi-component enzyme system and consists of three major components; endo-β-glucanase (EC 3.2.1.4), exo- β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Lynd *et al*., 2002; Bhat, 2000). Bacteria, due to their high natural diversity, faster growth have the capability to produce highly thermostable, alkali stable enzyme complement and may serve as highly potential sources of industrially important enzymes. *Bacillus* sp. continue to

be dominant bacterial work horses due to the capacity of some selected species to produce and secrete large quantities of extracellular enzymes (Aa *et al*., 1994; Singh *et al*., 2004). Reports of strains belonging to species such as *Bacillus sphaericus* and *Bacillus subtilis* which display high cellulose degradation activities (Mawadza *et al*., 1996; Singh *et al*., 2004). The production of extracellular cellulase in microorganisms is significantly influenced by a number of factors such as temperature, pH, aeration (Immanuel *et al*., 2006) and medium constituents. The relationship between these variables has a marked effect on the ultimate production of the cellulase. There are reports on the influence of various fermentation parameters on cellulase production by different bacteria (Immanuel *et al*., 2006; Rajoka, 2004). Major industrial applications of cellulases are in textile industry for 'bio-polishing' of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness (Cavaco-Paulo, 1998). They are also used in animal feed for improving the nutritional quality and digestibility, in processing of fruit juices and in baking, while de-inking of paper is yet another emerging application (Tolan and

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Foody, 1999). Another promising area of the cellulases is the bio-conversion of renewable cellulosic biomass to sugars for fermentation of bio-ethanol and bio-based products on a large scale (Cherry and Fidantsef, 2003). Current global revenues for goods produced using industrial biotechnology are estimated at between EUR 50 billion and EUR 60 billion annually, according to data released by industry trade publications. There are many predictions of future market values. For example, one estimate is that the global market for industrial biotechnology could reach roughly EUR 300 billion by 2030. Much of the activity has now been in bio-fuels, but there is also an established market for bio-based chemicals. While bio plastics. The capacity of selected Bacillus strains to produce and secrete large quantities (20-25 g/L) of extracellular enzymes has placed them among the most important industrial enzyme producers. The ability of different species to ferment in the acid, neutral and alkaline pH ranges, combined with the presence of thermophiles in the genus, has led to the development of a variety of new commercial enzyme products with the desired temperature, pH activity and stability properties to address a variety of specific applications. Bacillus species are attractive industrial organisms for a variety of reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete proteins into the extracellular medium and the Generally Regarded as Safe (GRAS). Status by the Food and Drug Administration for species, such as *B. subtilis* and *Bacillus licheniformis*. In addition, much is now known about the biochemistry, physiology and genetics of *B. subtilis* and other species which facilitates further development and greater exploitation of these organisms in industrial processes. The aim of this study was for the production of extracellular endoglucanase by a novel new strain of *Bacillus* sp.RL1 isolated from soil was improved by medium optimization.

MATERIALS AND METHODS

Screening and isolation of the organism: One gram of the soil sample (Jin Yun mountain, 800 m, China) was weighed and mixed in 10 mL of water which was serially diluted from 10^{-1} to 10^{-9} dilutionsterial-CMC-agar, plates containing (g/L), KH_2PO_4,2; $(NH_4)_2SO_4$, 4; $MgSO_4$, 0.5; Peptone, 10; agar, 20 and distilled water, supplemented with 1% carboxymethyl cellulose (CMC) pH-7.0. Plates were prepared and spread plate technique was done for isolating the colonies. The plates were incubated at 30ºC for 24 h for bacteria. After the incubation period the plates were flooded with Congo red along with NaCl which was used to detect the organism producing EG. The colonies that produced zone were selected and was purified and subcultured for other purpose. The bacterial isolate was identified using normal laboratory technique, Study of colony morphology of the isolated culture was carried out followed by gram"s staining and endospore staining.

Physical and Biochemical characterization of the isolated colonies was carried out using standard protocol (Kannan, 2003). Identification was carried out according to Berges"s Manual (7th Ed). The bacterial culture isolated was newly strain *Bacillus* sp.RL1.

Phylogenetic analysis: The 16S rRNA gene sequence of *Bacillus* sp.RL1 was determined by direct sequencing of the purified PCR-amplified 16SrRNA gene fragment as described previously by Kato *et al*. (2004). Genomic DNA was extracted by the CTBA protocol and was used as the PCR template. PCR was performed with universal bacterial primers complementary to conserved regions of the 5 and 3ends of the 16S rRNA gene, 27F (forward)(5-AGAGTTTGATCCTGGCTCAG-3 and 1492R (reverse) (5-GGTTACCTTGTTACGACTT -3.PCR was performed using AmpliTaq Gold (Applied Bio systems). The PCR products were purified with a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instruction. The purified 16S rRNA gene was sequenced directly using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and an ABI PRISM model 377 genetic analyzer (Applied Biosystems). The obtained 16S rRNA gene sequences of isolated bacteria were compared with those from the DDBJ nucleotide sequence database using the program BLAST.

Confirmation of endoglucanase activity: We design this protocol to in our laboratory to verify the EG production was confirmed with 0.5 ml of 1% substrate (CMC) suspended in 0.05 M Sodium citrate buffer (pH 4.8) was incubated with 0.1 ml supernatant free cell, at 50°C for 30 min. Then at 100°C for15 min, stopped with 0.1 ml 1% (v/v) α-naphthol added and shook gently.0.5 ml of concentrated H_2SO_4 added, the solution color should change to brown indicate positive result.

Enzyme production: *Bacillus* sp.RL1 was cultured in a medium that contained carboxy methyl cellulose (10 g/L), pepton (10 g/L), $(NH_4)_2SO_4$ (4 g/L), KH_2PO_4 (2 g/L) and $MgSO_4.7H_2O$ (0.5 g/L). The initial pH of the medium was adjusted to 7.0. Cultures were grown up until the beginning of stationary phase at 30°C. After removing the cells by centrifugation (Hettich Universal 30 RF) (10000 g, 10 min) at 4°C, the supernatant was used for further work.

Enzyme activity assay:
Endo- β-1,4-glucanase assay: EG activity was determined by incubation of 1ml of 1% CMC in (w/v) CMC dis-solved in 0.05 M sodium citrate buffer pH 4.8 with 1ml supernatant at 50°C. After 30 min reaction, 1mL of Dinitrosalicylic Acid (DNS) was added and boiled in a water bath for 15 min to stop the reaction. The resulted samples were then cooled to room temperature and measured the absorbance at 540 nm

(A540).One unit of endo-β-1,4-glucanase activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 µg of glucose within 1 min reaction at 50°C (Miller, 1959).

FPase assay: The activity of FPase was assayed according to the method explained by Gautam *et al.* (2010). This method is similar to the CMCase assay method, but the substrate was Whatman no. 1 filter paper strip (1x6 cm) soaked in 1 ml 0.05 M citrate buffer (pH 4.8). The samples were incubated with 1 ml enzyme solution at 50 C for 30 min, the reducing sugar liberated during growth were determined. One unit of FPase activity was determined as 1µmol of glucose liberated per ml enzyme per minute (Miller, 1959).

Xylanase assay: Xylanase activity was assayed using a 1% solution of oat spelt xylan as the substrate as described by Bailey *et al.* (1992) and the amount of reducing sugars released was determined by the dinitrosalicylic acid method (Miller, 1959). One unit of enzyme activity was defined as 1µmol xylose equivalent produced per minute under the given conditions.

Glucosidase assay: Glucosidase activity was measured by monitoring the release of glucose or reducing sugar from salicin (Gautam *et al.*, 2010). One unit of glucosidase activity is defined as the amount of enzyme required to catalyze hydrolysis of the substrate in 1 min to generate 1µmol glucose at pH 7.0 and 35°C. The above extracts were made an assay of the activities against carboxyl methyl cellulose (endo-glucanase), salicin (*B*-glucosidase) and Xylan (xylanase) at 35°C, respectively. All Data recorded in this study are average outcome of three replications plus standard error.

Optimization of culture conditions for enzyme production:

Effect of temperature on endoglucanase activity: In order to determine the effect of temperature on EG production and extracellular protein, the selected bacterial isolate was grown in CMC broth and incubated at 20, 35, 40, 45 and 50°C for 72h. Culture broths were then centrifuged at 10'000 rpm for 10 minutes to obtain supernatants which will be measured EG activity and extracellular protein.

Effect of the pH value on endoglucanase activity: The effect of initial media pH on EG production was conducted by adjusting the CMC broth to pH 5.0, 6.0, 7.0, 8.0 and 9.0 before bacterial inoculation. After 72 h of incubation at 35°C culture broths were then centrifuged at 10'000 rpm for 10 minutes to obtain supernatants which were later measured EG and activity and extracellular protein.

Thermo stability of endoglucanase: Thermostability of EG was determined by pre-incubation the enzyme solution at 30-100°C for 30 min before using it for assaying the residual activity. For determining pH stability, the enzyme was pre-incubated at 45°C with 0.05 M sodium citrate buffer pH 4.8 of varying pH 4.0-10 for 30 min and residual activity was analyzed.

Effect of incubation time on endoglucanase activity: *Bacillus* sp.RL1. was inoculated into production medium and incubated at 35°C for 120 h in stationary phase conditions. The enzyme activity was measured at regular intervals of 24 h.

Effect of carbon sources on endoglucanase activity: Under optimized temperature, pH and incubation period. Five different carbon sources (starch, manitol, xylose, sucrose and pineapple shell) were tested at the same concentration of 10g/l on EG production.

Effect of nitrogen sources on endoglucanase activity: Three different experiments were conducted to study the effects of the nitrogen source on the growth of *Bacillus s*p.RL1 and its production of EG in the CMC medium. In the first experiment, four different organic nitrogen sources were tested; 10g/L each of beef extract, urea, tryptone and yeast extract was separately added to the CMC medium. In the second experiment, four different inorganic nitrogen sources were tested; 4g/L each of L-glutamine, L- aspargine, calcium nitrate $Ca(NO_3)_2.4H_2O$ and sodium nitrate $(NaNO_3)$ was separately added to the CMC medium. The results of the first and second test led to a third test to study the effect of combined best organic nitrogen source beef extract 10g/l and best two inorganic nitrogen sources4g/l each of L-glutamine and sodium nitrate $(NaNO_3)$ was separately added to the CMC medium on EG production was studied.

Agitation speed: *Bacillus* sp.RL1. was inoculated into production medium and incubated at 35°C for 120 h in stationary phase conditions at three different conditions as follows; static, 100rpm and 200rpm using 50ml shaker flask. The EG activity was measured at regular intervals of 24 h.

Effect of metal ions on endoglucanase activity: The effects of various metal ions on EG activity was determined by pre-incubating the enzyme with the individual metal ions K^+,Ca^{2+},Li^+ and Co^{2+} and chemical reagent, phosphate sources ($NaHPO_4.2H_2O$, $Na_2HPO_4.12H_2O$ and K_2HPO_4), sulfate sources ($CuSO_4.5H_2O$, $ZnSO_4.5H_2O$ and Na_2SO_3) and detergents (SDS and EDTA) for 30 min at 45°C. Residual activity was measured using the enzyme assay, as described above. The activity assayed in the
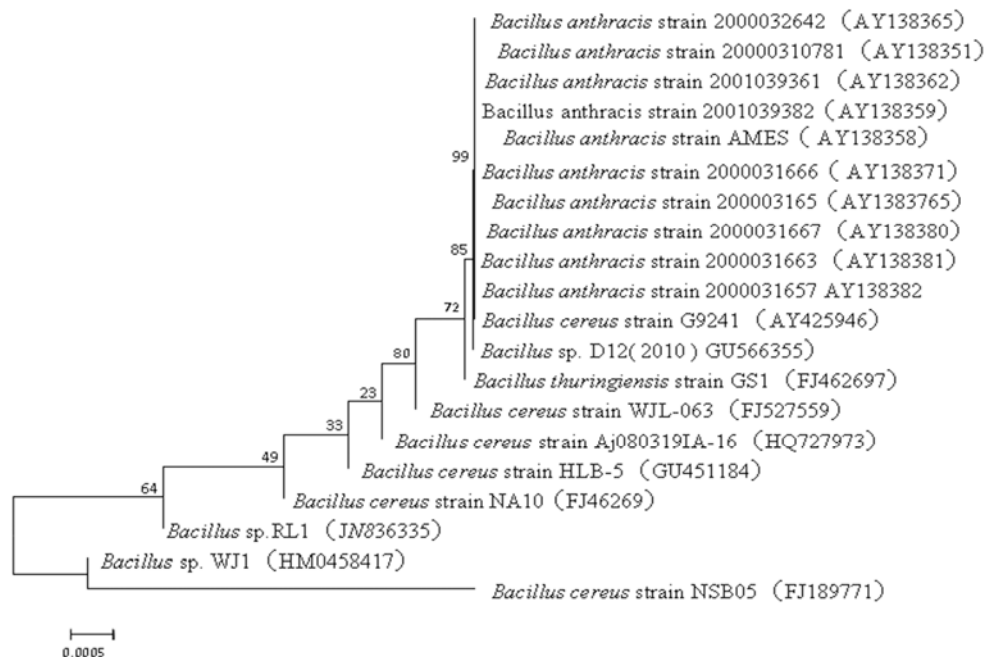
Fig. 1: Phylogenetic tree based on neighbor-joining method showing relationship of strain *Bacillus* sp.RL1 with closest representatives and type species. Bootstrap values 100 replications are shown at the branch. The tree was generated using Kimura 2-parameter model and Cantor correction

absence of metal ions was recorded as 100% (control). Each value is an average of triplicate tests plus standard error.

Determination of extra-cellular protein: The protein of the samples was estimated using the Bradford (1976) method with bovine serum albumin (A-4503, Sigma) as the protein standard.

Kinetic determinations: Initial reaction rates using CMC as substrate were determined at substrate concentrations of 0.5-10 mg/mL in 50M phosphate buffer (pH 7.5) at 35°C. The kinetic constants, Km and Vmax, were estimated using linear regression plots of Line weaver and Burk.

RESULTS

Screening and identification of microorganisms: A total of eight bacterial isolates were screened for endoglucanase production on CMC agar. Among the isolated strains, positive strain was selected by exposing the agar plates to Congo red solution (1%). The strain was aerobic, gram positive, rod, motile, starch analysis and spore forming. The maximum endoglucanase producer of the isolate newly strain *Bacillus* sp.RL1 was chosen for further investigation. The nucleotide sequence of the 16S rRNA gene of the isolate *Bacillus* sp.RL1 was also determined. And the gene sequence was deposited in the Gen Bank database with accession number JN 836335. The sequence had 1553bp and G+C content of the sequence was found at 53.46%. The 16S rRNA sequence analysis of *Bacillus* sp.RL1 revealed that the organism shared 99% of its identity with different Bacillus species such as *Bacillus cereus* HS-MP13, *Bcillusthuringiensis* BMB171, *Bacillus anthracis* AMES and *Bacillus* sp.4CC58 (Fig. 1).

Optimization of parameters for endoglucanase production: Media optimization is an important aspect to be considered in the development of fermentation technology. Formulation of media that is cost effective for the production of EG can reduce the cost of enzyme production. EG is an inducible and it is affected by the nature of the substrate used for production.

Effect of temperature on endoglucanase activity and stability: The effect of temperature on EG activity was determined at various temperatures ranging from 30 to 50°C. The cultivation temperature has a marked influence on endoglucanase activity as well as on extracellular protein, in current work the optimum temperature for endoglucanase activity and extracellulare protein was found to be 35°C with 0.0775 U/ml and 1.1548mg/ml respectively (Fig. 2a). While the thermal stability of EG was determined at various temperature from 30-90°C at pH 7.0, 85.5% of the original endoglucanase activity was maintained at temperature 90°C after 30min pre-incubation. More than 70% of the original endoglucanase activity was
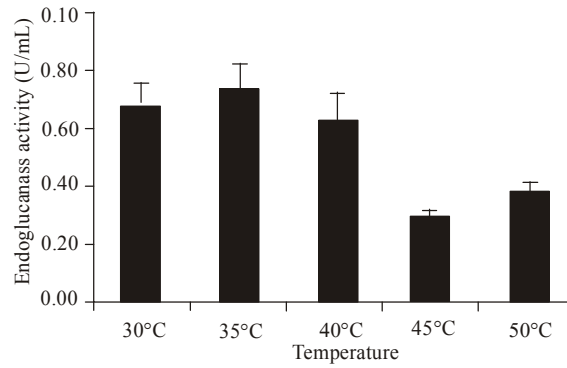
Fig. 2a: Effect of initial temperature on endoglucanase activity (U/mL) during fermentation with 1% CMC as substrate, pH 7.0
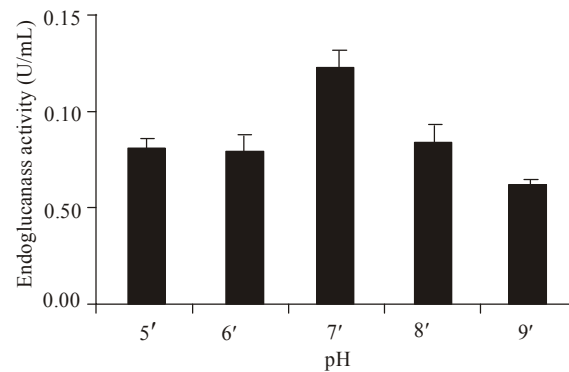

Fig. 2b: Effect of initial pH on endoglucanase activity (U/mL) during fermentation with 1% CMC as substrate, 35°C
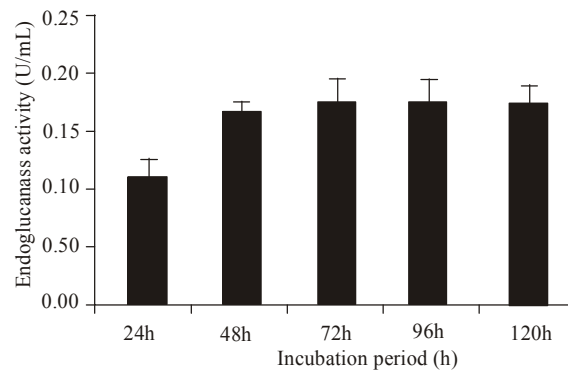

Fig. 2c: Effect of incubation period on endoglucanase activity (U/mL) during fermentation with 1% CMC as substrate, pH 7.0
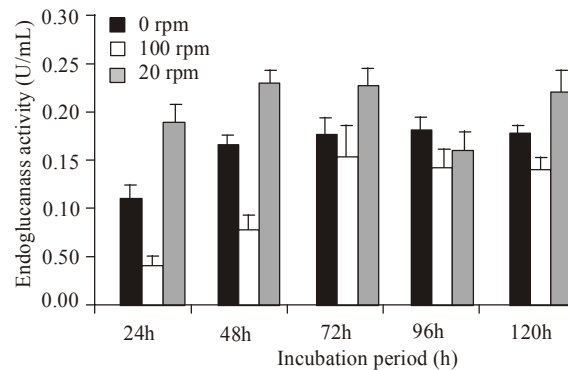

Fig. 2d: Effect of agitation speed on endoglucanase activity (U/mL) during fermentation with 1% CMC as substrate, pH 7.0, 35°C, static, 100rpm and 200rpm

maintained at abroad temperatures throughout the experiments from 30-90°C.

Effect of pH on endoglucanase activity and stability:
Optimization of pH demonstrated optimal EG activity 0.1295U/mL was observed at pH 7.0, IP 6.5 (Fig. 2b). The pH stability of the enzyme between pH 4.0 and 10 was determined by incubating the enzyme in 0.05mM sodium citrate buffer of different pH values at 45°C for 30 min and then assaying the residual enzyme activity under standard assay conditions at pH 6.0. EG was stable between pH 4.0 and 10. The stability over a broad pH range seems to be characteristic of many *Bacillus* endoglucanases. But it had maximum activity at pH 5.0.

Effect of incubation period on endoglucanase activity: The effect of incubation period on EG activity was estimated for 120h. The enzyme activity was found to increase steadily with an increase in incubation time. Maximum activity was observed at 72h 0.1852U/mL (Fig. 2c). While after 72h of incubation the enzyme activity decreased slightly.

Effect of agitation speed on endoglucanase activity:
With operating temperature and pH maintained at 35°C and pH 7.0 respectively, the effect of agitation speed was investigated by comparing the performance of the agitation rate at three agitation rates namely 0 rpm (static), 100 rpm and 200 rpm for 120 h. Results show a remarkable decrease in fermentation medium under shaking condition 100rpm compared to static and 200rpm conditions. Whereas the results reveal increase the activity of the enzyme under shaking condition 200rpm compared to static and 100rpm conditions. It was observed that EG activity (Fig. 2d).

Effect of carbon sources on endoglucanase activity:
Five types of carbon sources were used starch, manitol, xylose, sucrose and pineapple shell. Figure 3a shows the influence of carbon sources on EG production from

newly strain *Bacillus* sp.RL1. Maximum activity of EG was supported by xylose 2.5751U/mL after 72h of fermentation, considerably good enzyme activity was observed on starch 1.0441U/mL and pineapple shell 0.6120U/mL. It was quite interesting to observe that enzyme activity was significantly enhanced 4 folds on natural substrate of pineapple shell compared to that on pure substrate (CMC, control). The lowest endoglucanase activity was found in the medium with sucrose and manitol, 0.0840U/mL and 0.0710U/mL, respectively as a carbon sources.

Effect of nitrogen sources on endoglucanase activity:
To evaluate the effect of nitrogen sources on EG production from newly strain *Bacillus* Sp.RL1, three experiments were tested, in the first test the effect of four organic nitrogen sources was investigated, i.e., yeast extract, beef extract, urea and tryptone, Fig. 3b shows the production of the enzyme. Overall, the beef extract gave the best endoglucanase productivity 0.4939U/ml followed by yeast extract, tryptone and urea, 0.2608U/mL, 0.1999U/ml and 0.1841U/ml respectively. In the second test, the effect of four inorganic nitrogen sources was investigated, i.e., L-asparagine, L-glutamine, sodium nitrate (NaNO$_3$) and calcium nitrate Ca (NO$_3$)$_2$. 4H$_2$O, Fig. 3c reveal that the L-glutamine gave the best endoglucanase productivity 0.2101U/ml followed by NaNO$_3$ 0.1667U/ml. The lowest EG productivity was found in the medium with calcium nitrate Ca(NO$_3$)$_2$. 4H$_2$O and L-asparagine 0.1654U/ml and 0.0477U/ml respectively. The results of the first and second test led to a third test to combination of best organic nitrogen source beef extract plus the best two inorganic nitrogen sources, L-glutamine and NaNO$_3$. The results of the third test show that the combination of beef extract with second best inorganic nitrogen source NaNO$_3$ the EG productivity increase remarkably 4.5 folds 0.9280U/ml, whereas 3.5 fold increase in the case of a combination beef extract with L-glutamine 0.6248U/ml (Fig. 3d).
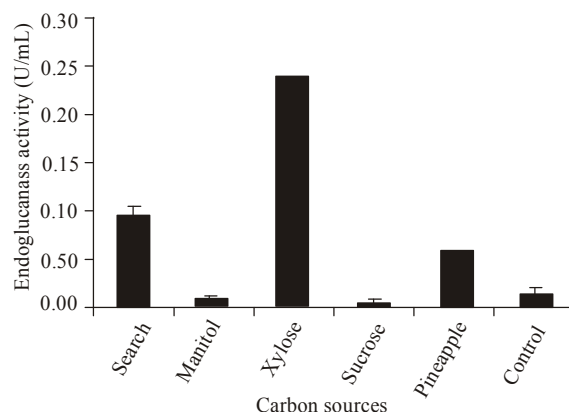

Fig. 3a: Effect of carbon sources (1%) on endoglucanase activity (U/mL) during fermentation with 1% different carbon sources as substrate, pH 7.0, 35°C, 72h
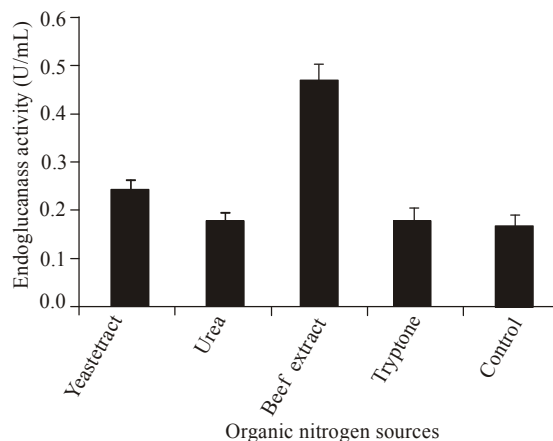
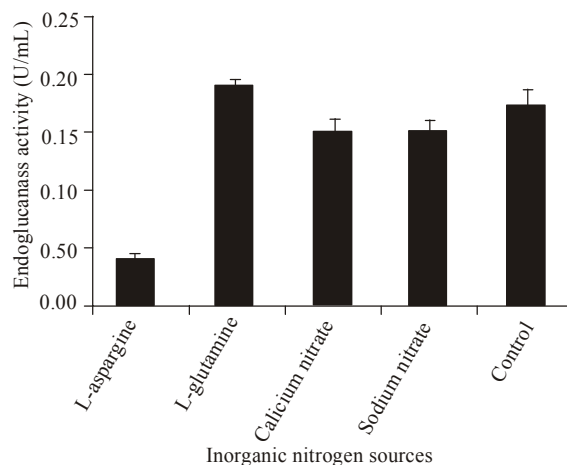Fig. 3b: Effect of organic nitrogen sources (1% w/v) on endoglucanase activity (U/mL)

Fig. 3c: Effect of inorganic nitrogen sources (0.4% w/v) on endoglucanase activity (U/mL)
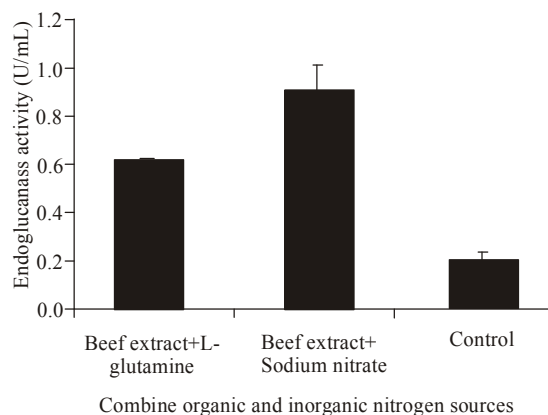

Fig. 3d: Effect of combined best organic (1% w/v) and inorganic (0.4% w/v) nitrogen sources on endoglucanase activity (U/mL)

Effect of metal ions and chemical reagents on endoglucanase activity: The effect of various ions and surfactants (SDS and EDTA) on EG productivity was shown in Fig. 4. In Fig. 4a, $FeSO_4.7H_2O$ and CO^{2+} ions caused a significant increase on enzyme activity 0.5099U/mL and 0.2922U/mL respectively. Most metal ions and chemical reagents such as phosphate sources

($NaHPO_4.2H_2O$, $Na_2HPO_4.12H_2O$ and K_2HPO_4) sulfate sources $CuSO_4.5H_2O$, $ZnSO_4.5H_2O$ and Na_2SO_3 and detergents SDS and EDTA, have slightly influenced on enzyme activity.

Comparative between basil medium (CMC) and optimized medium: The results revealed Fig. 4b effect
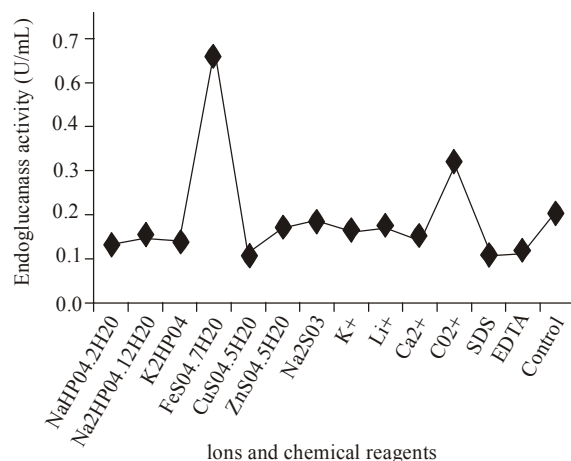
Fig. 4a: Effect of different ions and chemical reagents on endoglucanase activity (U/mL) during fermentation with 1% CMC as substrate, pH 7.0, 35°C, 72h
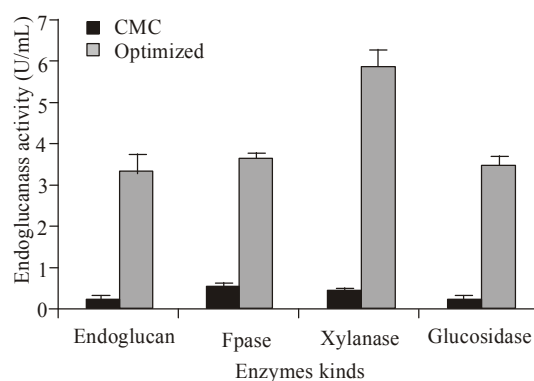

Fig. 4b: Comparison between basil medium (CMC) and optimized medium on enzymes activity (U/mL) during fermentation with 1% CMC as substrate, pH 7.0, 35°C, 72h

Table 1: Nutrients of basil medium (CMC) and optimized medium

Nutrients	CMC-Na medium	Concentration (%)	Optimized medium	Concentration (%)
Carbon source	CMC	1	Pineapple	1
Organic nitrogen source	Peptone	1	Beef extract	1
Inorganic nitrogen source	(NH4)2SO4	0.4	Sodium nitrate	0.4
Sulfur source	MgSO4.7H2O	0.05	MgSO4.7H2O	0.05
			FeSO4.7H2O	0.02
Phosphate source	KH2PO4	0.2	KH2PO4	0.2

of medium composition on enzymes activity from newly strain *Bacillus* sp.RL1. Grown on CMC agar, EG, FPase, Xylanase and glucosidase. 0.1852U/mL, 0.5642U/mL, 0.4522U/mL and 0.2496, respectively. Figure 4b shows a significant increase on enzymes activity when *Bacillus* sp.RL1. cultured in medium contain natural carbon source pineapple shell 1%, beef extract 1%, sodium nitrate 0.4%, $FeSO_4.7H_2O$, 0.02%, KH_2PO_4, 0.2%, $MgSO_4.7H_2O$, 0.05% (Table 1). EG, FPase, Xylanase and glucosidase, 3.4142U/mL (18 folds), 3.6758U/mL (6.5 folds), 5.8788U/ml (13 folds) and 3.4300U/mL (14 folds) respectively.

DISCUSSION

Bacillus species have been major work horse industrial microorganisms with roles in applied microbiology, which date back more than a thousand years (Schallmey *et al.*, 2004). EG from microorganisms is one of the key enzymes on account of their industrial applications (Ohmiya *et al.*, 1997; Bhat, 2000; Kapoor *et al.*, 2010). EG production of different organisms has received more attention and exploited for generation of cellulolytic enzymes with the use of cheaply available lignocelllosic residues (Lakshmikant *et al.*, 1990; Kaur and Satyanarayana, 2004; Chandra *et al.*, 2007; Kapoor *et al.*, 2010). The endoglucanase production by newly strain *Bacillus* sp.RL1. Was supported by different carbon sources among of them pineapple shell, which is cheap and available carbon source. The high cost of enzymes production has hindered the industrial applications of endoglucanase. The use of cheap carbon sources are important as these can significant reduce cost of

enzymes production; our findings stated that pine apple shell caused a significant increase of EG. Some investigators showed that use alternative substrates particularly crude raw materials of agricultural origin have been explored ac cost-effective substrates. CMC production medium was replaced with alternative substrates such as wheat straw, wheat bran, saw dust, rice bran, rice straw, sugar cane bagasse. It was quite interesting to observe that enzymes activity was significantly enhanced on all of these crude substrates as compared to that on pure CMC medium (Heck *et al.*, 2002; Nizamudeen and Bajaj, 2009; Kapoor *et al.*, 2010; Shabeb *et al.*, 2010). The EG showed activity over a wide range of pH 5.0-9.0 with optimum pH 7.0 and pH stability over pH 4.0-10, similar findings were reported by several other workers (Bakare *et al.*, 2005; Immanuel *et al.*, 2007; Dutta *et al.*, 2008; Bijende *et al.*, 2009). EG produced by newly *Bacillus* sp.RL1. was the gradual increase with maximum level observed at 72 h of incubation at 35℃ beyond 72h of incubation the enzyme production substantially decreased, probably due to the depletion of essential nutrients in the media and/or accumulation of toxic secondary metabolites produced by the bacterium itself.

Generally microbial EG from different organisms have been found to have temperature optimum of 35-50°C (Bakare *et al.*, 2005; Immanuel *et al.*, 2007; Ariffin *et al.*, 2008; Bijende *et al.*, 2009; Nizamudeen and Bajaj, 2009). Clearly endoglucanase in this study was active in a large spectrum of pH and temperature conditions. This was in correlation with the findings of many other workers (Mawadza *et al.*, 2000; Ariffin *et al.*, 2006; Immanuel *et al.*, 2006; Das *et al.*, 2010). Our results clearly stated that the EG activity produced using newly strain *Bacillus* sp.RL1. was higher three folds when supplement CMC medium with beef extract 0.4939U/ml followed by yeast extract 1.4folds, these results are in agreement with previous reports by Ariffin *et al.* (2008), Gao *et al.* (2008) and Kapoor *et al.* (2010). The productivity of EG recorded at that conditions was refer to importance of organic nitrogen source it has vitamins and other growth precursors, from the data obtained (Fig. 2d) it is best supported the importance of agitation in fermentation conditions to facilitate the homogenous condition and results in this study was in correlation with findings of many other workers (Khan and Husaini, 2006; Odeniyi *et al.*, 2009). The current study also elucidates the kinetic parameters of endoglucanase the Km values obtained using CMC as substrate from newly *Bacillus* sp.RL1. Were lower than those observed for *Lenzitestrabea* (Christakopoulos *et al.*, 1999), *Melanocarpussp* (Kaur *et al.*, 2007) and *Salinivibriosp*strain NTU-05 (Wang *et al.*, 2009). The response of the endoglucanase activity to metal ions seemed to follow a pattern resembling other *Bacillus* endoglucanases CO^{+2} caused significant increase (Au and Chan, 1987; Okoshi *et al.*, 1990; Yoshimatsu *et al.*, 1990; Lusterio *et al.*, 1992;

Mawadza *et al.*, 2000). Carbon source plays an important role in cellulase production. This is because the enzyme is induced based on the type of carbon source used. High cost of substrates can be a limiting factor in enzyme production.

CONCLUSION

These studies us to conclude that the microorganisms may serve as a good source of different microbial enzymes like Endoglucanase, FPase, Xylanase and glucosidase. Those organisms could be used for large scale production of enzymes under optimized culture conditions.

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