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Isolation, Screening and Optimization of *Geobacillus stearothermophilus* Cellulase Production using Date Palm Cellulosic Wastes

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Cellulases have a potential for use in biotechnology and industry such as in starch processing, alcoholic beverage manufacture, malting and brewing, clarification of juice, pulp bleaching, textile manufacturing and animal feed production. Thirty one thermophilic and cellulolytic bacteria were successfully isolated from a southern region of Saudi Arabia. The phylogenetic analysis of the promising isolate using its 16S rDNA sequence data showed its highest homology (99%) with *Geobacillus stearothermophilus*. The analysis was performed to evaluate cellulase production using untreated and both alkaline and acid-steam treated date palm wastes as carbon sources. The optimal conditions for cellulase production consisted of a 2.5% alkaline-treated date palm leaf base with pH at 7.0 - 7.5, a cultivation temperature at 45-50°C, an agitation rate of 200 rpm, and 0.2% Tween 80 and yeast extract as a best nitrogen source when fermentation was extended up to 48h. The results indicated that the utilization of date palm waste as a cheap substrate for the production of cellulases had potential, and could reduce environment pollution.

Key words: Isolation, 16S rDNA, *Geobacillus stearothermophilus*, cellulases, date palm.

The conversion of low-value agriculture residues using saccharification and fermentation processes into valuable commodities, energy, chemicals and microbial protein is not currently economically feasible, largely due to the costs of cellulosic materials and cellulolytic enzymes, as well as technical problems associated with cellulose saccharification[1]. A new approach to reducing the cost of enzyme production is proposed using agriculture waste as a carbon source, in addition to reducing the problems of environmental pollution. Saudi Arabia (SA) is a major date-producing country, and is ranked second in the world as per FAO statistics (2010). The total area planted with date palm trees in Saudi Arabia was about 162,000 hectares, with approximately 23 million palm trees made up of more than about 400 varieties[2]. Date palm trees produce large quantities of agricultural waste which can be utilized as an economical source of carbohydrates for the industrial production of cellulases and glucose syrup, the main source for the production of high value products[3].

Cellulases are among the industrially-important hydrolytic enzymes, and are of great significance in present day biotechnology. Cellulases are widely used in the food, feed, textile and pulp industries. The bioconversion of cellulosic materials is now a subject of intensive research, as a contribution to the development of large scale conversion processes beneficial to mankind[4]. The industrial demand for thermostable

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cellulase enzymes continues to stimulate the search for thermophilic microorganisms that can be used to produce sufficient amounts of these enzymes. Bacteria which have a high growth rate as compared to fungi have good potential to be used in cellulase production[5,6,7]. The high cost of cellulase production with low enzyme activities limits its industrial use. Therefore, efforts are needed to economize cellulase production by media optimization[8].

Cellulase production has been influenced by a number of factors including environmental and nutritional conditions; pH, temperature, agitation, nitrogen sources, carbon concentration and surfactants. The relationship between these variables has a marked effect on the production of the cellulase enzymes[9]. The present research will result in new uses, and in expanding markets for the inexpensive agricultural residuals of date palm for the production of important value-added products and for providing a new method for reducing the problem of agricultural wastes. The main objectives of this investigation were the isolation of thermophilic cellulase-producing bacteria from the Asser region of Saudi Arabia and their screening for cellulase production. In addition, it is hoped to identify promising isolates by 16S rDNA sequence technique. Furthermore, it is hoped to optimize the factors affecting cellulase production using treated and untreated date palm wastes as inexpensive carbon sources.

MATERIALS AND METHODS

Samples collection

Soil samples were collected (April, 2015) from the Jazan (JA), Abha (AB), Najran (NA), Bisha (BI), Mahail (MA), Tanoma (TA) and Nammas (NM) regions of southern Saudi Arabia.

Treatments of date palm waste

Date palm cellulosic waste (leaves, leaf bases and fibers) were collected from a date palm plantation in Saudi Arabia, and used as the carbon substrate in this study. Ground substrates were treated by two methods: (1) Alkaline pre-treatment: 2N NaOH at 30°C for 48 h[10]; (2) Acid-steam pre-treatment: 1% H_2SO_4, 120°C for 100 min[11]. After treatment, the waste was washed thoroughly with tap water until neutralization took place, then oven dried at 70°C and crushed before use.

Isolation and screening for cellulase-producing thermophilic bacteria

Two protocols were used for the isolation of thermophilic cellulases producers: (A): Direct isolation; the collected soil samples were suspended and serially diluted in sterile distilled water up to 10^{-4}. 1.0 ml of each dilution and water sample (without dilution) were spread on agar plates and incubated at 50°C for 1-2 weeks. The plates were stained with Congo Red and scored to create a clear halo surrounding the colony[12]. (B): Enrichment producers: Cellulase-producing thermophilic bacteria were isolated by the enrichment of water and soil samples using cellulosic sources. Samples of 5 g soil $5ml^{-1}$ water were added to 250 ml Erlenmeyer flasks containing 95 ml of Bushnell Haas medium (BHM), supplemented with 5.0% different cellulosic substrates as the sole carbon source, cellulose, CMC and cellobiose[13]. The enrichment cultures were incubated at 50ºC and 200 rpm for 2-3 weeks. The BHM medium consisted of: $MgSO_4 \cdot 7H_2O$, (0.2 gl^{TM1}); K_2HPO_4, (1 gl^{TM1}); KH_2PO_4, (1 gl^{TM1}); NH_4NO_3, (1.0 gl^{TM1}); $FeCl_3 \cdot 6H_2O$, (0.05 gl^{TM1}); $CaCl_2$, (0.02 gl^{TM1}). The cultures were diluted in sterile distilled water, plated on nutrient agar medium, and incubated at 50ºC for 2 d. The purified colonies were screened for their cellulolytic ability using BHM supplemented with different substrates as the sole carbon source. After incubation for 2 d at 50°C, the plates were flooded with 1% Congo Red for 15-20 mins, and then washed with 1 M NaCl solution. A clear zone that formed around the growing colonies of cellulase positive cultures was taken as the indication of cellulase activity[12]. Promising colonies having a significant clear zone in all three media were selected for further study.

Bacterial genomic DNA extraction

The genomic DNA of each strain was isolated according to[14]. Cells were collected from overnight LB cultures by centrifugation, and re-suspended in 500 µl TEN buffer. Twenty five µl of 10 mg ml^{-1} of lysozyme were added, and the tubes were incubated at 37 ºC for 30 mins, followed by the addition of 75 µl of 10% SDS. The tubes were then inverted gently several times until complete lysis. Three µl of 20 mg ml^{-1} of Proteinase K were added, and the tubes were incubated at 37ºC for one h. After incubation, 100 µl of NaCl (5M) were added, followed by 800 µl of phenol/chloroform:

isoamyl alcohol (24:1). The tubes were then inverted several times and then centrifuged for 10 min. The upper phase was transferred to a fresh sterile tube and extracted once with chloroform. The upper phase was again transferred to a fresh tube and 0.7 volume of isopropanol was added and mixed gently and centrifuged for 10 mins. The supernatant was removed carefully, and the pellets were washed with 1 ml of 70% ethanol. The pellets were collected by centrifugation for 5 min. The DNA was dried and dissolved in 100 µl TE buffer and stored at -20°C.

Amplification and sequencing of the 16S rDNA gene

The 16S rDNA gene was amplified by polymerase chain reaction (PCR) using specific universal primers[15]. The sequence for the 16S primer for 16S1500F was 5'-AGAGTTTGATCMTGGCTCAG-3' and the sequence for the 16S primer for 16S1500R was 5'-AAGGAGGTGWTCCARCC-3'. The PCR mixture consisted of 30 pico moles of each primer, 10 ng of chromosomal DNA, 200 µM dNTPs and 2.5 units of Taq polymerase with 10 µl of polymerase buffer containing $MgCl_2$. The PCR was carried out for 10 min at 94°C, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, followed by a final extension step at 72°C for 10 min. After completion, a fraction of the PCR mixture was examined using 1.5% agarose gel in a TBE buffer (pH 8.5). Electrophoresis was carried out for 20 min at 150 V. The 16S rRNA gene PCR product was sequenced by MACROGENE, Korea. The homology of the 16S rRNA gene sequence of the isolates with reference to 16S rRNA sequences was analyzed using the BLAST algorithm in Gen Bank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Production of cellulases using promising isolates using date palm waste

The promising isolate was further screened for their cellulase (Endoglucanase, Exoglucanase and b-glucosidase) production using treated and untreated date palm waste. A loopfull of culture from the agar plate was inoculated into a 50 ml tube containing 5 ml of nutrient broth, and incubated at 50°C/200 rpm. This culture was then used to inoculate a 250 ml Erlenmeyer flask containing 50 ml production medium using 1% treated and untreated date palm waste as the sole carbon source. The cultures were incubated at 50°C for 48 h. The cells and insoluble materials were removed by centrifugation at 10,000 for 10 min and the cell-free supernatants were used for enzyme assay. Various process parameters influencing cellulase production were optimized. The effect of the fermentation period (up to 96 h), the incubation temperature (35 to 60°C), the initial pH (pH 6 to 9), the agitation rate (0-300 rpm) the substrate concentration (0.5-4%), the nitrogen sources and the surfactants were investigated.

Enzymes assay

Exoglucanase activity (FPase) was measured according to[16]. The reaction mixture contained 0.5 ml of 50 mM acetate buffer at pH 5. A 0.5 ml culture filtrate and one filter paper strip (1×3 cm) were used as the crystalline cellulose substrate. The mixture was incubated for 60 min at 50°C. The reaction was terminated using the 1 ml DNS (3,5-dinitrosalicylic acid) method[17], boiled for 5 min, and cooled in water for color stabilization. The optical density was measured using a double beam UV/Vis scanning spectrophotometer (Model: Shimadzu, 1601PC) at 540 nm. The cellulase activity was determined by using a calibration curve of glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1µ mole of glucose.

Endoglucanase activity (CMCase) was measured using the above-mentioned method for the determination of FPase activity, except that 0.5 ml of 1% carboxymethylcellulose (CMC) solubilized in 50 mM acetate buffer, pH 4.8 was used as a substrate. The mixture was incubated for 20 mins at 50°C. One unit of enzyme activity was defined as the amount of enzyme that released 1µ mole of glucose.

β-Glucosidase activity was assayed with 0.02% P-nitrophenyl-β-D-glucopyranoside (PNPG) as a substrate in 50 mM acetate buffer, pH 5.0. A reaction mixture containing 10µml culture filtrate and completed to 1 ml with buffered substrate were incubated at 50°C. The reaction was terminated after 10 mins with 3 ml 0.1M NaOH. The absorbance was measured at 400 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1.0 µM P-nitrophenol[18].

Statistical analysis

Analyses of data were done by analysis of variance (ANOVA) using the MSTATC program. The Least Significant Difference (LSD) at p d" 0.05 was used to detect differences among treatments.

RESULTS AND DISCUSSION

Isolation and screening of cellulases producing thermophilic bacteria

The bacterial strains present an attractive potential for the utilization of cellulases due to their rapid growth rate, enzyme complexity and extreme habitat variability[19]. No cellulases producing bacteria could be isolated using the direct plating method without a pre-enrichment step. The same observation was reported by[20]. One hundred and sixteen bacterial isolates were obtained from soil samples enriched with cellulose, CMC and cellobiose. The results clearly indicate that the highest number of bacteria were isolated from soil samples from the Jazan (28), Najran (25) and Tanoma (17) regions, while low number of isolates were collected from soil samples of the Bisha (13), Nammas (10), Mahail (12) and Abha (11) regions. The occurrence of isolates in the different localities may be affected by the prevailing environmental conditions and nature of the soil.

All different isolates were screened for their ability to produce extracellular cellulases using the Congo Red test as reported by[20,21]. Seventy-nine isolates showed positive results with a clear zone around the colonies in one or more substrates. Only thirty one isolates gave a clear zone in all media, which indicates their ability to produce all three enzymes responsible for the conversion of cellulose to glucose[22]. The highest numbers of cellulolytic bacteria were isolated from soil samples of the Jazan (8), Najran (9) regions, with low number in samples from the Tanomma (3), Mahail (1), Bisha (3), Abha (5) and Nammas (2) regions. The highest cellulolytic ability was detected in isolate; NA-75 (2.6-3.6 cm) isolated from the Najran region (**Fig. 1**). [23]reported that thermophiles are adapted to survive in high temperature regions. These microorganisms produce unique biocatalysts that function under extreme conditions that are comparable to those prevailing in various industrial processes. Also, [24,1,7] isolated *Bacillus sp* using cellulosic waste as a carbon source.

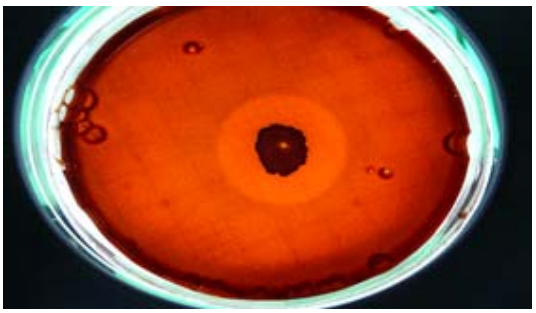

Fig. 1. Cellulose hydrolysis using promising bacterial isolate, NA- 75.

Molecular identification of the selected bacterial isolate using 16S rRNA gene

For bacterial identification, the genomic DNA was subjected to PCR amplification using universal primers. These primers were able to amplify about 1500 bp from the 16S rRNA gene. The 1500 bp PCR product was purified from the agarose gel and subjected to DNA sequencing. The DNA nucleotide sequence obtained was analyzed using DNA Blast search (NCBI). The results revealed the isolate NA-75 as *Geobacillus stearothermophilus* with a similarity of 99%.

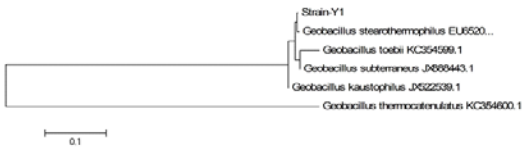

Fig. 2. Phylogenetic tree for *Geobacillus stearothermophilus* strain Y-1 and related species constructed by the neighbor-joining method based on 16rRNA gene sequences. Segments corresponding to an evolutionary distance of 0.1 are shown with bars. Accession numbers for sequences are as shown in the phylogenetic tree

Cellulases production by *Geobacillus stearothermophilus* using treated and untreated date palm cellulosic wastes as carbon sources

To investigate the possibility of reducing cellulolytic enzymes production cost, the effect of 1% untreated and both alkaline and acid-steam treated date palm waste in the form of leaves, leaf bases and fibers as inducing materials and carbon sources were studied. The enzyme production was carried out for 48h at 50°C, 200 rpm and pH 7. The results of CMCase, FPase and b- glucosidase production bacterial isolate were as illustrated in Fig. (3). The results indicate that the cellulases produced using pretreated waste was significantly higher than that using untreated ones. The most efficient carbon source for cellulase production was the alkaline treated leave base with the

maximum enzymes production being 5.03 Uml^{-1} CMCase, 3.56 Uml^{-1}FPase and 1.82 Uml^{-1}b-glucosidase.

The alkaline pre-treatment of lignocellulosic materials caused the swelling of cellulose, leading to an increase in the internal surface area, a decrease in crystallinity, the separation of structural linkage between lignin and carbohydrates, and the disruption of the lignin structure[25,26]. The same trends in terms of results were also observed using treated and untreated date palm fibers. As mentioned in Fig. (2), untreated leaves were an optimum carbon source for FPase production, amounting to 1.49 Uml^{-1}. Furthermore, alkaline-treated date palm leaves were the best carbon source for CMCase and b-glucosidase production. This variation may be attributed to the chemical nature and nutrient availability of the substrates used. These findings were in agreement with those reported by[27]. They found that the delignified materials were not as efficient as the non-delignified substrates. This implies that lignin content does not inhibit cellulase production. The inhibitory effect of delignification on the cellulae production may be explained by structural changes in the cellulose during pretreatment, thereby reducing the availability of the materials as a carbon source.

Effect of fermentation period on cellulases production

The fermentation process was carried out up to 96 h and cellulases production was measured at 12 h intervals. The results illustrated in Fig (4) revealed that the most significant increase of enzymes production was obtained at 48 h yielded 3.50 Uml^{-1} FPase, 5.11 Uml^{-1} CMCase and 1.74 Uml^{-1} b-glucosidase, respectively. Maximum cellulases production was consistent with the growth trend; enzymes production was low during the lag phase to the mid-logarithm phase of growth, while the peak of enzymes production significantly occurred at the beginning of stationary phase. Similar findings have been reported[28,29,30,31]. The decreasing of cellulases production after optimum period may be due to nutrient exhaustion which stressed the microbial physiology resulting in the inactivation of secretary machinery of the enzymes and autolysis of microbial cells as reported by[32]. On the other hand, [33]explained the reduction in cellulase production by glucose produced in a culture medium which caused an inhibitory effect in terms of feedback inhibition of the enzyme activity.

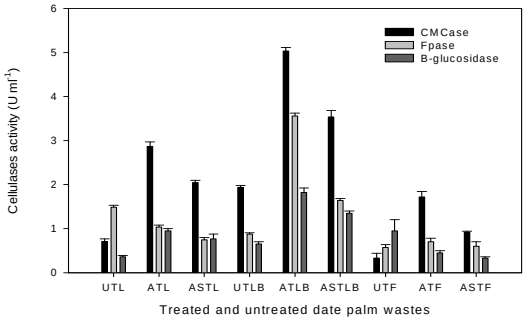

Fig. 3. Cellulase production by *Geobacillus stearothermophilus* using treated and untreated date palm waste as carbon sources (UTL: Untreated leaves, ATL: Alkaline treated leaves, ASTL: Acid-steam treated leaves, UTLB: Untreated leaf base, ATLB: Alkaline treated leaf base, ASTLB: Acid-steam treated leaf base, UTF: Untreated fibrous, ATF: Alkaline treated fibrous, ASTF: Acid-steam treated fibrous).

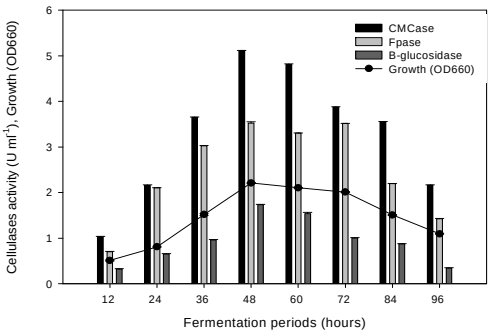

Fig. 4. Effect of fermentation period on cellulase production

Effect of initial pH on cellulases production

The initial pH of the medium influences many enzymatic systems and the transport of several enzymes across the cell membrane. The effect of pH was determined by adjusting the initial pH in the range of pH 6.0 to 9.0. The results show that the cellulase production was dependent on the pH values Fig (5). Maximum levels of FPase (4.36 Uml^{-1}) and CMCase (6.11 Uml^{-1}) were achieved significantly at 7.5, while a peak of β-glucosidase (1.80 Uml^{-1}) production, without significant effect, was obtained at pH 7.0. Beyond these pH values, cellulase production was markedly decreased.

These results were in the range reported by[30]. He reported that pH 7.0 was more suitable for endoglucanase production by *Geobacillus stearothermophilus* using sugarcane bagasse, and that it exhibited good pH stability between "5-8" and "4-9", for UntSCB and TSCB, respectively. Also, [34]reported that *Bacillus* sp. SM3M8 gave maximum cellulase production at pH 7.0. Furthermore, [35]recorded that at pH 6.5, *Bacillus subtilis* and *Erwinia* spp recorded optimal yields of cellulases, while *Serratia* spp and *Clostridium cellobioparum* recorded the same cellulolytic activities at pH 7.0. Moreover, [36,37]reported that the optimum pH for cellulase production by *Cellulomonas flavigena* and *Bacillus pumilus* was pH 6. Also, [38,39]reported that the cellulase production from *Streptomyces* sp. and *S. ruber* was active over a pH range of 5-7 with maximum activity at pH 6. In contrast, [40]found that maximum cellulolytic activities were recorded at pH 8 for *Bacillus circulans* and *B. megaterium*.

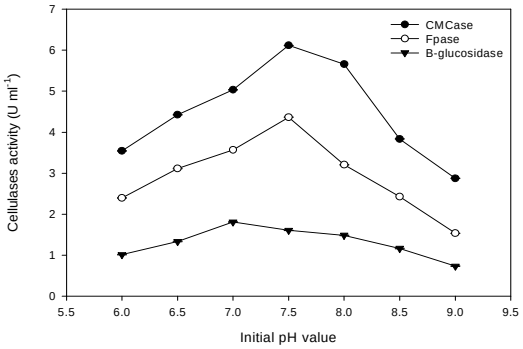

Fig. 5. Effect of initial pH on cellulases production.

Effect of incubation temperature on cellulases production

To determine the effective temperature for cellulase production, a fermentation process was carried out at 5°C intervals in the range of 35 to 60°C (Fig. 6). The results indicate a significant relationship between enzyme production and incubation temperature up to 45-50°C, followed by a gradual decrease. The cellulase activity at 55°C was also comparable; it lost 18.6%, 11.56% and 26.66% for FPase, CMCase and β-glucosidase activity, respectively. These results were in agreement with those reported by[41,42,30]. They recorded 50°C as the optimal temperature for cellulase production by *Streptomyces* T3-1, *Bacillus subtilis* and *Bacillus agaradherens* JAM-Ku-23; and *Geobacillus stearothermophilus* respectively. In addition, [38]found that the maximum CMCase activity of *Streptomyces* sp. was recorded at 60°C with no significant difference between 50 and 60°C.

Many researchers have reported different temperatures for maximum cellulase production, suggesting that the optimal temperature depends on the strain variation of the microorganism. [35]reported that the cellulolytic activities of tested bacterial isolates peaked at 38.6°C although there was no significant difference among the individual values recorded. They reported that at 35°C, *Serratia* spp, *Bacillus subtilis* and *Bacillus circulans* recorded optimal cellulolytic activities, while *Clostridium thermocellum* however attained optimal cellulolytic activity at 50°C. Also, [34]reported that the *Bacillus* sp. SM3M8 gave maximum cellulase production and activity at a temperature of 45°C, while [35,39]reported that 40°C was optimal for the endoglucanase activity of *Bacillus* spp and *Streptomyces ruber*. Furthermore,[40]recorded maximum cellulolytic activities of *Bacillus circulans* and *B. megaterium* at 30°C. On the other hand, [43,36]recorded that maximum cellulase activity of *Bacillus pumulus* EB3 and *Cellulomonas flavigena* were at 37°C and 55°C, respectively.

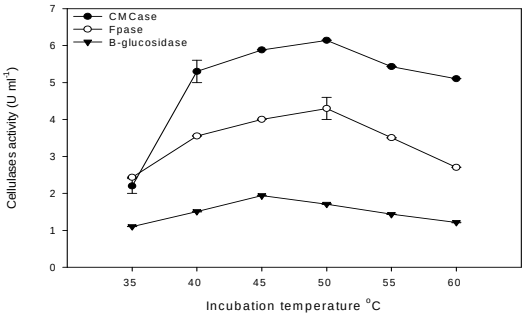

Fig. 6. Effect of incubation temperature on cellulase production

Effect of agitation rate on cellulase production

The effect of agitation rate on cellulase production was investigated at 0.0, 100, 150, 200, 250 and 300 rpm. The results illustrated in Fig (7) showed a significant increase in cellulase production due to agitation. Maximum cellulase production was significantly elucidated at 200 rpm,

which may be due to the increase in the aeration ratio. Furthermore, increasing the agitation rate above an optimum value was unfavorable for cellulase production except for the CMCase which insignificantly maintained their good production value up to 250 rpm. Under static conditions, the enzyme production decreased to 18.30%, 30.16% 24.29% for FPase, CMCase and b-glucosidase, respectively.

These results were in agreement with those reported by[44,45,46]. They found that the cellulolytic enzymes in static conditions were not significant and that the high concentration of carbon source results in an increased viscosity of the medium, that affects the availability of oxygen needed for microbial growth. They also reported that agitation is important for proper oxygen transfer and for the homogenous mixing of nutrients in the fermentation system. A study of cellulase production in a bioreactor by[47] concluded that agitation maintains a concentration gradient between the interior and exterior of the cell, allowing a better diffusion of nutrients to the cells. They found also that enzyme deactivation increased with an increase in agitation speed.

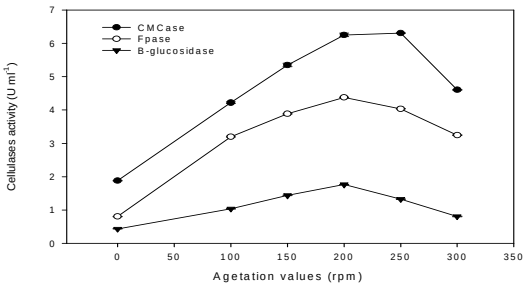

Fig. 7. Effect of agitation rate on cellulase production.

Effect of nitrogen sources on cellulase production

To evaluate the effect of alternative nitrogen sources on cellulase production, ammonium nitrate was replaced by organic and inorganic nitrogen sources in such amounts that the final nitrogen concentration in the media remained unchanged. The results illustrated in Fig (8) revealed that all nitrogen sources stimulated cellulase production. Optimum cellulase production was significantly obtained using yeast extract as a nitrogen source. The most significant increase of FPase, CMCase and b-glucosidase production were 36.51%, 32.80% and 53.75%, respectively over a control medium which was significant when compared to other inorganic sources. The superior effect of complex organic nitrogen compounds could be due to the presence of growth promoters in sufficient amounts to cover the requirements of microbial growth and enzyme production. The same observations were reported by[48,37]. Various inorganic nitrogen sources have been optimized by different workers for cellulase production[49,50].

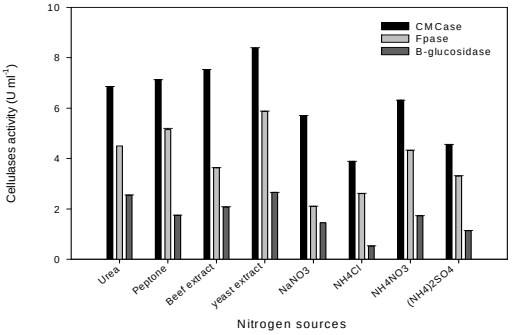

Fig. 8: Effect of nitrogen sources on cellulase production

Effect of alkaline treated date palm leaf base (ATLB) concentration on cellulase production

Cellulases are relatively costly enzymes, and a significant reduction in cost will be important for their commercial uses. For an economic point of view, there is a need to increase cellulase production by using cheaper substrates such as agriculture waste[13]. The different concentrations of a selected carbon source, alkaline-treated date palm leaf base (ATLB), was examined to identify the best for maximal cellulase production. As shown in Fig. (9), the bacterial isolate has an ability to metabolize the cellulosic substrate for growth by expressing cellulolytic enzyme activities. The cellulose production was increased gradually in proportion to an ATLB concentration of up to 2.5% which gave significant production in the form of 7.54 Uml^{-1} FPase, 11.13 Uml^{-1} CMCase and 3.71 Uml^{-1} β-glucosidase. The enzyme activity decreased to about half of the maximum at 3%.

The decreasing cellulase production at higher substrate concentrations can be explained by lignin inhibition and an increase in mass transfer resistance as reported by[26]. Also, [51]reported that the reduction of cellulase production at high substrate concentrations may be due to the production of reducing sugars, which significant inhibits the enzyme activity during cellulose

hydrolysis. Agricultural waste at different concentrations have been used for cellulase production. [39]found that 30 gl^{-1} rice straw was the most suitable concentration for maximum cellulase production using *Streptomyces ruber.* Furthermore, [28,52]used acid-hydrothermal treated oil palm empty fruit bunch fibers and corncobs as carbon sources at 10 g L^{-1} for cellulase production. Also, [30]revealed that the presence of 60 and 50 g L^{-1} sugarcane bagasse supported maximal avicelase production for both strains of *Geobacillus stearothermophilus*, UntSCB and TSCB, respectively.

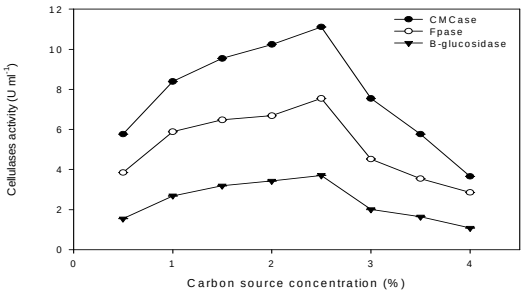

Fig. 9. Effect of carbon source (ATLB) concentration on cellulase production

Effect of surfactants on cellulase production

To investigate the effect of surfactants on cellulase production, three surfactants were tested, Trix-100, Tween-80 and SDS (Sodium Dodecyl Sulphate) at 0.2 and 0.5%. The results illustrated in Fig (10) indicate that Tween 80 at 0.2% was a good stimulant for cellulase production. The significant increase in cellulase production compared with a control medium was recorded as 11.82%, 16.62% and 13.53% for FPase, CMCase and b-glucosidase, respectively. The activity effect of surfactants on cellulase production using bacterial strains has been observed by many investigators[53] reported that surfactants are amphiphilic compounds which can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids and increasing the solubility, mobility, bioavailability and subsequent biodegradation of hydrophobic or insoluble organic compounds which act to increase enzyme yield. [37]reported that the maximum cellulase production using *Bacillus pumilus* EWBCM1 was recorded in a Tween-20 added medium, while the minimum cellulase production was recorded in SDS. On the other hand, [53]reported that the Tween 80 increased the activities of amylase, protease, CMCase and xylanase to different extents, while rhamnolipid only had an obvious stimulatory effect on xylanase production by *Streptomyces badius*.

CONCLUSION

Thermophilic cellulase producing bacteria, *Geobacillus stearothermophilus* was isolated from the Najran region of Saudi Arabia. Maximum cellulase production was obtained using 2.5% alkaline treated date palm leaf base as a carbon source, and yeast extract as the best nitrogen source. Optimum cellulase production was achieved when the fermentation period was extended to 48 h at an initial pH value (7.0 -7.5) and incubation temperature (45-50 °C) with a 200 rpm agitation rate and 0.2% Tween 80. The results of this work indicate that date palm cellulosic waste can be used as a cheap carbon source for the isolation and production of cellulase enzymes.

REFERENCES

1. Rahna, K., Rathnan, D., Balasaravanan, T. Isolation screening, identification and optimized production of extracellular cellulase from Bacillus subtilis using cellulosic waste as carbon source. *J. Microb.* 2013; **2**: 2383-2386.
2. El-Habba, M., Al-Mulhim, F. The competitiveness of the Saudi Arabian date palm. An analytical study. *Afr. J. Agri.* 2013; **8**:5260-5267.
3. Wang, C., Shyu, C., Ho, S. Characterization of a novel thermophilic cellulose degrading bacterium – *Paenibacillus spp* strain B39. *Lett. Appl. Microbiol.* 2008; **47**: 46 - 53.
4. Behera, B., Parida, S., Dutta, S., Thatoi, H. Isolation and identification of cellulose degrading bacteria from mangrove soil of Mahanadi river delta and their cellulase production ability. *Amer. J. Microb. Res.* 2014; **2**: 41-46.
5. Sonia Sethi, Aparna Datta, B. Lal Gupta, and Saksham, Gupta. Optimization of Cellulase Production from Bacteria Isolated from Soil. Hindawi Publishing Corporation, ISRN Biotechnology, 2013; Article ID 985685.
6. Khianngam, S., Pootaeng-on, Y., Techakriengkrai, T., Tanasupawat, S. Screening and identification of cellulase producing bacteria isolated from oil palm meal. *J. Appli. Pharm. Sci.*, 2014; **4**: 090-096.

7. Ladeira, S., Cruz, E., Delatorre, A., Barbosa, J., Martins, M. Cellulase production by thermophilic *Bacillus* sp. SMIA-2 and its detergent compatibility. *Electro. J. Biotech.* 2015; **18**: 110-115.
8. Sethi, S., Lal, D. and Gupta, S. Optimization of cellulase production from bacteria isolated from soil. *Biotechnology*, 2013, Article ID 985685, 7 pages.
9. Patagundi, B., Shivasharan, C., Kaliwal, B. Isolation and Characterization of Cellulase producing bacteria from Soil. *Int. J. Curr. Microbiol. App. Sci.* 2014; **3**(5): 59-69.
10. Deshpande, M. Ethanol production from cellulose by coupled saccharification fermentation using Saccharomyces cerevisiae and cellulose complex from *Sclerotium rolfsii* UV-8 mutant. *Appl. Biochem and Biotech.* 1992; **36**: 227-234.
11. Carrasco, J., Saiz, M., Navarro, A., Soriano, P., Saez, F., Martinezm, J. Effect of dilute acid and steam explosion pretreatments on the cellulose structure and kinetics of cellulosic fraction hydrolysis by dilute acids in lignocellulosic materials. *App. Bioch. Biot.* 1994; **45**: 23-34
12. Wood, T., Bhat, K. Methods for measuring cellulose activities. *Methods Enzymol*. 1988; **160**: 87-112.
13. Lo, Y., Saratalea, G., Chen, W., Bai, M., Chang, J. Isolation of cellulose-hydrolytic bacteria and applications of the cellulolytic enzymes for cellulosic biohydrogen production. *Enz. Microb. Techn*. 2009; **44**: 417- 425.
14. Sambrook, J., Fritsch, E., Maniatis, T. Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, 1989; N.Y.
15. Poppert, S., Essig, A., Stoehr, B., Steingruber, A., Wirths, B., Juretschko, S., Reischl, U., Wellinghausen, N. Rapid diagnosis of bacterial meningitis by real-time PCR and fluorescence in situ hybridization. *J. Clin. Microbiol*. 2005; **43**: 3390-3397.
16. Miller, G. Use of dinitrosalycilic acid reagent for determination of reducing sugar. *Anal. Chem.* 1959; **31**: 426-428.
17. Reczey, K., Stalbrand, H., Presson, I., Hahm-Hagerdal, B., Tjerneld, F. Continuous cellobiose hydrolysis using self-immobilized ß-glucosidase from *Aspergillus phoenicis* QM329 in a fluidized bed reactor. *Appl. Bioch. Biotech.* 1990; **24**: 637-649.
18. Maki, M., Leung, K., Quin, W. The prospect of cellulase producing bacteria for bioconversion of lignocellulosic biomass. *Int. J. Bio. Sci.* 2009; **5**: 500-516.
19. Ibrahim, A., El-diwany, A. Isolation and identification of new cellulases producing thermophilic bacteria from an Egyptian hot spring and some properties of the crude enzyme. *Austral. J. Basic Appl. Sci.* 2007; **1**: 473-478.
20. Aboul-Enein, A., Abou-Elalla, F., Serour, E., Hussien, T. Molecular identification of novel thermophilic actinomycetes isolated from Egypt. *Int. J. Acad. Res.* 2009; **1**: 80-84.
21. Yi, J., Sandra, A., Shu, T. Production and distribution of endoglucanase, cellobiohydrolase, and b-glucosidase components of the cellulolytic system of *Volvariella volvacea*, the edible straw mushroom, *Appl. Environ. Microbiol*. 1999; **65**: 553-559.
22. Haki, G., Rakshit, S. Developments in industrially important thermostable enzymes: a review. *Bioresour. Technol.* 2003; **89**: 17-34.
23. Kowsalya, R., Ramasamy, K. Isolation, screening and characterization of cellulase producing Bacillus subtilis KG10 from virgin forest of Kovai Kutralam, Coimbatore, India. *Res. J. Biotech.* 2013; **8**: 17-23.
25. Damisa, D., Ameh, J., Umoh, V. Effect of chemical pretreatment of some lignocellulosic wastes on the recovery of cellulase from *Aspergillus niger* AH3 mutant. *Afr. J. Biotechnol.* 2008; **7**: 2444-2450.
26. Reczey, K., Szengyel, Z., Eklund, R., Zacchi, G. Cellulase production by *T. Reesei. Bioresour. Technol.* 1996; **57**: 25-30.
27. Sudto, A., Punyathiti, Y., Pongsilp, N. The use of agricultural wastes as substrates for cell growth and carboxymethyl cellulose (CMCASE) production by Bacillus subtilis,Escherichia coli and Rhizobium sp. *Kmitl Sci. Tech. J.* 2008; 8: 84-92.
28. Bajaj, B., Pangotra, H., Wani, M., Sharma, P., Sharma, A. Partial purification and characterization of a highly thermostable and pH stable endoglucanase from a newly isolated Bacillus strain M-9. *Indian J. Chem. Technol*. 2009; **16**: 382-387.
29. Makky, E. Avicelase production by a thermophilic Geobacillus stearothermophilus isolated from soil using sugarcane bagasse. *World Acad. Sci. Engin. Tech.* 2009; **57**:487-491.
30. Mukesh Kumar, D., Poovai P., Puneeth Kumar, C., Sushma Saroja, Y., Manimaran, A., Kalaichelvan, P. Optimization of *Bacillus cereus* MRK1 cellulase production and its Biostoning activity. *Der Pharm. Lett.* 2012; **4**: 881-888.
31. Chellapandi, P., Himanshu, M. Production of endoglucanase by the native strains of *Streptomyces* isolates in submerged

fermentation. *Braz. J. Microbiol.* 2008; **39**: 122-127.

32. Demerdash, M. Adsorption of *Aspergillus niger* cellulase enzymes on cellulosic substrate during fermentation. *J. Agric, Sci. Mansoura Univ.* 1997; **22**: 719-728.
33. Rasul, F., Afroz, A., Rashid, U., Mehmood, S., Sughra, K., Zeeshan, N. Screening and characterization of cellulase producing bacteria from soil and waste (molasses) of sugar industry. *Internat. J. Biosci.* 2015; **6**: 230-238.
34. Immanuel, G., Dhanusha, R., Prema, P., Palavesam, A. Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. *Internat. J. Environ. Sci. Tech.* 2006; **3**: 25-34.
35. Perez-Avalos, O., Sanchez-Herrera, L., Ponce-Noyola, T. Bifunctional endoglucanase - endoxylanase from *Cellulomonas flavigena* with potential use in industrial processes at different pH. *Curr. Microbiol.* 2008; **57**: 39 - 44.
36. Shankar, T., Isaiarasu, L. Cellulase production by *Bacillus pumilus* EWBCM1 under varying cultural conditions. *Middle-East J. Sci. Res.* 2011; **8**: 40-45.
37. Jaradat, Z., Dawagreh, A., Ababneh, Q., Ismail, S. Influence of culture conditions on cellulase production by *Streptomyces* sp. (Strain J2). *Jordan J. Biol. Sci.* 2008; **1**: 141-146.
38. El-Sersy, N., Abd-Elnaby, H., Abou-Elela, G., Ibrahim, H., El-Toukhy, N. Optimization, economization and characterization of cellulase produced by marine Streptomyces ruber. *Afri. J. Biotech.* 2010; **9**: 6355-6364.
39. Sangbriba, R., Duan, C., Tang, J. Isolation and characterization of mesophilic *Bacillus* species cellulase genes from black liquor. *Bioresour. Technol.* 2006; **14**: 2727 - 2733.
40. Hirasawa, K., Uchimura, K., Kashima, M., Grant, W., Ito, S., Kobayashi, T., Horikoshi, K. Salt activated endoglucanase of a strain of alkaliphilic *Bacillus agaradhaerens. Antonie Van Leuwenhoek.* 2006; **89**: 211-219.
41. Li, W., Zhang, W., Yang, M., Chen, Y. Cloning of the thermostable cellulase gene from newly isolated *Bacillus subtilis* and its expression in *Escherichia coli*. *Mol. Biotech.* 2008; **2**: 195-201.
42. Ariffin, H., Hassan, M., Shah, U., Abdullah, N., Ghazali, F., Shirai, Y. Production of bacterial endoglucanase from pretreated oil palm empty fruit bunch by *Bacillus pumilus* EB3. *J. Biosci. Bioeng.* 2008; **106**: 231-236.
43. Najafpour, G.D.: Gas and Liquid System (Aeration and Agitation). In: *Biochemical Engineering and Biotechnology* (Najafpour GD, ed). Netherlands: Elsevier, 2007; pp: 22-68.
44. Nadeem, M., Qazi, J., Baig, S. Effect of aeration and agitation rates on alkaline protease production by *Bacillus licheniformis* UV-9 mutant. *Turk. J. Biochem.* 2009; **34**: 89-96.
45. Fadzilah, K., Mashitah, M. Cellulases production in palm oil mill effluent: effect of aeration and agitation. *J. Appl. Sci.* 2010; **10**: 3307-3312.
46. Xu, C., Yun, J. Influence of aeration on the production and the quality of the exopolysaccharides from Paecilomyces tenuipes C240 in a stirred-tank fermenter. *Enzy. Microbial. Technol.* 2004; **35**: 33-39.
47. Gautam, S., Bundela, P., Pandey, A., Jamaluddin, Awasthi M., Sarsaiya, S. Optimization of the medium for the production of cellulase by the *Trichoderma viride* using submerged fermentation. *Internat. J. Environ. Sci.*, 2010; **1**: 656-665.
48. Lee, B., Kim, B., Lee, Y., Chung, C., Lee, J. Industrial scale of optimization for the production of carboxymethylcellulase from rice bran by marine bacterium, *Bacillus subtilis* subsp. subtilis A53. *Enzy. Microbiol. Techn.* 2010; **48**: 38-42.
49. Sherief, A., Tanash, A., Atin, N. Cellulase production by *Aspergillus fumigatus* grown on mixed substrate of rice straw and wheat bran. *Res. J. Microb.* 2010; **5**: 199-211.
50. Sangkharak, K., Vangsirikul, P., Janthachat, S. Isolation of noval cellulase from agricultureal soil and application for ethanol production. *Intern. J. Advan. Biotech. Res.* 2011; **2**: 230-239.
51. Shahriarinour, M., Wahab, M., Ariff, A., Rosfarizan, M. Screening, isolation and selection of cellulolytic fungi oil palm empty fruit bunch fiber. *Biotech.* 2011; **10**: 108-113.
52. Singh, A., Van Hammeb, J., Warda, O. Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotech. Advan.* 2007; **25**: 99-121.
53. Liu, J., Shi, J., Li, J., Yuan, X. (ed): Effects of surfactants Tween 80 and rhamnolipid on the extracellular enzymes amylase, protease, CMCase and xylanase of one strain. International Conference on Computer Distributed Control and Intelligent Environmental Monitoring, Cdciem, 2011; pp 1207-1210.