# Optimization and Physiochemical Properties of Xylanase from *Bacillus Coagulans* and *Bacillus Licheniformis*

Mohamed I. Abou-Dobara, Ahmed Kasem El-Sayed and Reham A. El Fayoumy *

Botany Department (Microbiology), Faculty of Science, Mansoura University (Da mietta Branch), New Damietta, Egypt. *reham85@mans.edu.eg

**Abstract:** Xylanase production by *Bacillus coagulans* and *Bacillus licheniformis* was optimized. Maximum xylanase production could be achieved after an inc ubation period of 48 hrs, at 50°C and pH 6 for *Bacillus coagulans and* after an incubation period of 60 hrs, at 50°C and pH 7 for *Bacillus licheniformis.* Xylan (0.2%) was found to be the best carbon source among the tested carbohydrates. *Bacillus coagulans* grew well and produced high level of xylanase using ammonium sulfate as nitrogen source but peptone was the best nitrogen source for producing high level of xylanase for *Bacillus licheniformis*. The properties of xylanase enzyme were tested. Km (mg/ml) and Vmax (µmole/ml/min) for *Bacillus coagulans* was found 3.0 and 0.641 respectively but for *Bacillus licheniformis* was found 4.0 (mg/ml) and 0.568 (µmole/ml/min). For thermal stability for *Bacillus coagulans*, the enzyme lost 50 % of the activity in (6 hours and 42 minutes) and (6 hours and 54 minutes) at 50°C and 60°C respectively, but at 70°C and 80°C the half life time of xylanase enzyme at was 7 hours. And for *Bacillus licheniformis* the enzyme lost 50% of the activity in 7 hours, (7 hours and 12 minutes) and (7 hours and 42 minutes) at 50°C, 60°C and 70°C respectively, but at 80°C the half life time of xylanase was 8 hours.
The best temperature for crude enzyme of *Bacillus coagulans* was found to be 50°C but for *Bacillus licheniformis* was found to be 70°C. The best pH for crude enzyme of *Bacillus coagulans* was found 6 but for *Bacillus licheniformis* was found 8.
[Mohamed I. Abou-Dobara, Ahmed Kasem El-Sayed and Reham A. El Fayoumy. Optimization and Physiochemical Properties of Xylanase from *Bacillus Coagulans* and *Bacillus Licheniformis.* Journal of American Science 2011; 7(12):661-670]. (ISSN: 1545-1003). http://www.americanscience.org.

**Keywords:** optimization, xylanase, *Bacillus coagulans* and *Bacillus licheniformis.*

## 1. Introduction

Xylans are heterogeneous polysaccharides with a backbone consisting of beta-1,4 linked D-xylosyl residues. Depending on the source of xylan, substitutes such as arabinofuranosyl, 4-O-methylglucuronosyl and acetyl groups can be present at varying frequencies (Lynch et al., 1981). Xylan ranks second only to cellulose in abundance and comprises up to one third of the total dry weight of higher plants (Lynch et al., 1981). Endo-beta-1,4 xylanases (beta-1,4-D-xylanxylanohydrolase; EC3.2.1.8) are the main enzymes responsible for cleavage of the linkages within the xylan backbone (Gilbert et al., 1993). Complete hydrolysis of xylan to monomers involves beta-xylosidases (beta-1,4-D-xylosidexylohydrolase; EC 3.2.1.37) and various debranching enzymes as Acetyl xylan esterase (EC 3.1.1.6), Alpha arabinosidases (EC 3.2.1.99) and Alpha-glucuronidases (EC 3.2.1.-) (Gilbert et al., 1993).

Many bacteria and fungi have been studied for xylanase production (Shoham et al., 1992; Bailey et al., 1993; Morales et al., 1995; Angelo et al., 1997; Kar et al., 2006; Yang et al., 2006; Anuradha et al., 2007). There are several applications of xylanases in industries (Prade, 1996; Kulkarni et al., 1999; Subramaniyan and P. Prema, 2000). Currently the major applications of xylanases are in paper, feed and baking industries. Optimization studies in which one factor is varied at a time might lead to misinterpretation of the results. On the contrary, statistically planned experiments prove to be a useful tool for optimization of the factors affecting the objectives. Their applications for medium optimization, particularly with respect to xylanase production, have been established (Haltrich et al., 1993; Haltrich et al., 1994; El-Helow and El-Ahawany, 1999). The aim of this study is to optimize medium conditions for increasing the activity production of xylanase in free cell cultures of *Bacillus coagulans* and *Bacillus licheniformis.* Also the properties of crude enzyme were studied.

## 2. Materials and Methods

This study was conducted throughout the years 2008, 2009, 2010 and 2011 and carried out in the laboratories of Botany Department, Faculty of Science, New Damietta, Mansoura University, Egypt.

*Bacillus coagulans* and *Bacillus licheniformis* were isolated from muchroom compost collected from Egypt and identified according to Bergey's Manual of systematic bacteriology (Sneath, 1985).

The organisms were grown in 250 ml flasks, each containing 20 ml of Sorenson broth medium with the following composition (g/l): xylan, 2.0; $NH_4NO_3$, 1.0; $K_2HPO_4$, 0.5; NaCL, 0.2;

$MgSO_4.7H_20$, 0.5; $FeSO_4.6H_2O$, 0.02; distilled water 1000 ml. The pH was adjusted to 7.0. The flasks were inoculated with 1 ml of bacterial suspension obtained from nutrient agar cultures and incubated at 50°C with shaking at 150 rev $min^{-1}$ for 72 hrs. The filtrate was then separated by centrifugation at 5000 rpm for 10 min.

**Estimation of xylanase**

Xylanase was assayed by measuring the amount of reducing sugar released from 1% xylan. The amount of reducing sugar was estimated according to method of Miller (1959), in which the amount of the reducing sugars liberated was estimated following the 3, 5 dinitrosalicylic acid (DNS). The reaction mixture containing 1 ml of 1 % (w/v) xylan plus 0.1 ml of cultural supernatant and 0.9 ml distilled water. The mixture was incubated at 50°C for 20 min, centrifugated at 5000 rpm for 10 min. and aliquot of supernatants were assayed for reducing sugars by using 1 ml 3, 5 dinitrosalicylic acid (DNS). One unit of the enzyme activity was defined as the amount of enzyme that released 1 µmole of the reducing sugar (expressed as xylose equivalent) per milliliter per minute under assay conditions. Enzyme and substrate controls were included routinely.

**Growth estimation**

Growth was determined spectrophotometrically by measuring optical density of the culture at 600 nm. The final pH was also recorded at the end of some experiments.

**Factors affecting xylanase producing by *Bacillus coagulans* and *Bacillus licheniformis***

(1) Incubation period

The basal medium containing 0.2 % xylan was used; 20 ml medium in 250 ml conical flasks were inoculated with 1 ml of bacterial suspension in triplicate manner. The culture was incubated in an incubator shaker rate of 150 rpm. Samples were taken at 12 hrs intervals (12, 24, 36, 48, 60 and 72).

(2) Effect of different nitrogen sources

Different nitrogen sources were added to the medium according to the nitrogen content of ammonium nitrate (1gm per litter). These nitrogen sources included peptone, beef extract, yeast extract, casein, $(NH_4)_2SO_4$, $KNO_3$, and $NaNO_3$. The media were inoculated and incubated at 50°C for 48 hrs for *Bacillus coagulans* and for 60 hrs for *Bacillus licheniformis.*

(3) Effect of different carbon sources

Different carbon sources including starch, carboxymethylcellulose, cellulose, xylose, glucose, xylan, sucrose and lactose, were separately incorporated into flasks containing the production medium to the final concentration of 0.2% (w/v). The media were inoculated and incubated at 50°C for 48 hrs for *Bacillus coagulans* and for 60 hrs for *Bacillus licheniformis.*

(4) Effect of different concentration of xylan

Different concentration of xylan added to the media (0.2%, 0.4%, 0.6%, 0.8%, 1.0% and 1.2%). The media were inoculated and incubated at 50°C for 48 hrs for *Bacillus coagulans* and for 60 hrs for *Bacillus licheniformis.*

(5) Effect of different temperatures

Temperature effect was carried out by inoculating the flasks containing xylanase production media at pH 7 with the tested strains and incubated at different temperatures, notably 30, 35, 40, 45, 50, 55 and 60°C for 48 hrs for *Bacillus coagulans* and for 60 hrs for *Bacillus licheniformis.*

(6) Effect of different pH

Different flasks of basal xylanase production media of different initial pH values (3, 4, 5, 6, 7, 8, 9 and 10) were prepared. The pH was adjusted by using 0.1 N NaOH or 0.1 N HCl. The media were inoculated and incubated at 50°C for 48 hrs for *Bacillus coagulans* and for 60 hrs for *Bacillus licheniformis.*

**Physiochemical properties of the xylanase**

(1) pH

To study the effect of pH on the activity of xylanase, citrate phosphate buffer with pH's (from 3 to 5), phosphate buffer with different pH's (from 6 to 8), and carbonate buffer with pH 9 and 10, were used in the standard assays. Aliquots of the enzyme preparations were mixed with the different buffers at each pH and the enzyme activity was assayed as previously described. Activities were expressed as a percentage of the maximal activity.

(2) Temperature

Reaction mixtures were incubated at different temperatures (30, 40, 50, 60, 70 and 80°C) for 20 min, while the assay was carried out under the standard assay methods. Activities were expressed as percentage of the maximal activity.

(3) Thermal stability

The effect of the temperature on the stability of xylanase was determined by incubation the enzyme solution in the absence of substrate for different times at (50, 60, 70 and 80°C). The residual activity was assayed as described before.

(4) Km and Vmax

The Km and Vmax of xylanase were determined by varying the substrate concentration from 1.0 to 15 mg of xylan per ml. Data were plotted by the method of Lineweaver-Burk plot (Lineweaver et al., 1934).

**Statistical analysis:**

The experiments were carried out in three replicates with standard deviation and standard error presented. The statistical procedure used where appropriate was lest significant difference (LSD) using one way variance (ANOVA), software system was SPSS version 11 was used for analysis these results.

**3. Results**

The time course for xylanase production by *Bacillus coagulans* and *Bacillus licheniformis* in the basal liquid medium containing 0.2% xylan as substrate is shown in figure 1 and 2. The xylanase activity of both strains increased significantly (LSD at 0.05 Level) during the growth phase of the culture and the optimum production (2.068 U/ml) was reached after 48 hrs for *Bacillus coagulans*, but for *Bacillus licheniformis* the optimum production (1.19 U/ml) was reached after 60 hrs.

Thereafter, enzyme production declined extremely significantly (LSD at 0.05 Level) reaching the minimum level (0.53 U/ml) and (1.125 U/ml) for *Bacillus coagulans* and *Bacillus licheniformis* respectively after 72 hrs. For *Bacillus coagulans* maximum growth (0.16) was measured after 48 hrs and the growth declined extremely significantly (LSD at 0.05 Level) to reach the minimum level (0.08) after 72 hrs, but for *Bacillus licheniformis* maximum growth (0.165) was measured after 60 hrs and the growth declined extremely significantly (LSD at 0.05 Level) to reach the minimum level (0.1) after 72 hrs.

The best nitrogen source for xylanase production from *Bacillus coagulans* was ammonium sulfate (3.1370 U/ml) then decrease significantly (LSD at 0.05 Level) by using ammonium nitrate (2.3214 U/ml). The maximum growth measured (0.2433) with ammonium sulfate as shown in table (1).

The best nitrogen source for xylanase production from *Bacillus licheniformis* was peptone (2.0077 U/ml) then decrease significantly (LSD at 0.05 Level) by using ammonium nitrate (1.694 U/ml) and the maximum growth measured (0.17233) with peptone as shown in table (2).

*Bacillus coagulans* and *Bacillus licheniformis* were able to grow in basal liquid medium supplied with the different carbon sources. Which were starch, carboxymethylcellulose, cellulose, xylose, glucose, xylan, sucrose and lactose (table 3). The best carbon source for xylanase production and growth for *Bacillus coagulans* and *Bacillus licheniformis* was xylan with activity (2.4468 U/ml and 1.6563 U/ml respectively). Xylose, glucose, sucrose and lactose were also found to be good sources for the production of xylanase but using these sugars make the activity of xylanase decline significantly (LSD at 0.05 Level) compartion to using xylan as carbon source. Little growth was observed with carboxymethylglucose, glucose and xylose.

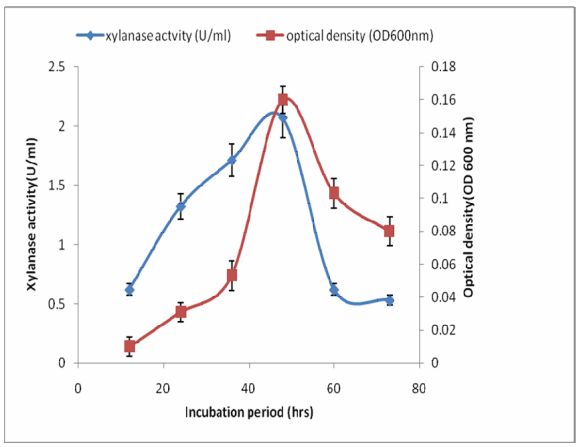


Fig (1): Effect of different incubation periods on xylanase production and growth of *Bacillus coagulans*.

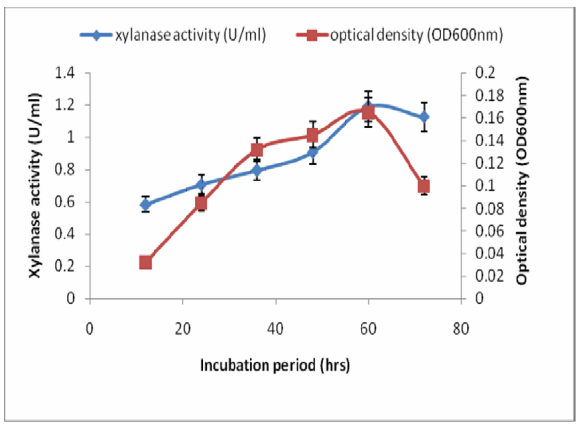


Fig (2): Effect of different incubation periods on xylanase production and growth of *Bacillus licheniformis.*

Table (1): Effect of different nitrogen sources on xylanase production and growth on *Bacillus coagulans* and *Bacillus licheniformis*

| Nitrogen Source | *Bacillus coagulans* | | | *Bacillus licheniformis* | | |
|---|---|---|---|---|---|---|
| | Final pH | Optical density (OD600nm) | Xylanase activity (U/ml) | Final pH | Optical density (OD600nm) | Xylanase activity (U/ml) |
| Peptone | 6 | 0.091 ± 0.0005 | 0.985 ± 0.010 | 8.45 | 0.172 ± 0.001 | 2.008 ± 0.0007 |
| Beef extract | 5.79 | 0.072 ± 0.001 | 0.621 ± 0.005 | 8.39 | 0.053 ± 0.001 | 0.408 ± 0.0007 |
| Yeast extract | 5.36 | 0.096 ± 0.0008 | 1.136 ± 0.004 | 7.1 | 0.092 ± 0.0008 | 0.959 ± 0.0007 |
| Casien | 5.15 | 0.153 ± 0.001 | 1.669 ± 0.003 | 8.71 | 0.122 ± 0.0003 | 1.418 ± 0.006 |
| $(NH_4)_2SO_4$ | 5.07 | 0.243 ± 0.001 | 3.137 ± 0.003 | 5.7 | 0.132 ± 0.001 | 1.443 ± 0.003 |
| $KNO_3$ | 4.45 | 0.121 ± 0.0005 | 1.456 ± 0.007 | 5.86 | 0.096 ± 0.001 | 0.985 ± 0.002 |
| $NH_4NO_3$ | 6.1 | 0.23 ± 0.0003 | 2.321 ± 0.003 | 5 | 0.152 ± 0.001 | 1.694 ± 0.006 |
| $NaNO_3$ | 5.59 | 0.113 ± 0.001 | 1.349 ± 0.003 | 5.6 | 0.085 ± 0.0008 | 0.903 ± 0.001 |

Table (2): Effect of different carbon sources on xylanase production and growth on *Bacillus coagulans and Bacillus licheniformis*

| Carbon Source | *Bacillus coagulans* | | | *Bacillus licheniformis* | | |
|---|---|---|---|---|---|---|
| | Final pH | Optical density (OD600nm) | Xylanase activity (U/ml) | Final pH | Optical density (OD600nm) | Xylanase activity (U/ml) |
| Starch | 6.4 | 0.163 ± 0.001 | 0 ± 0 | 5.57 | 0.344 ± 0.002 | 0.144 ± 0.005 |
| Cmc | 6.19 | 0.022 ± 0.0007 | 0.094 ± 0.008 | 5.37 | 0.031 ± 0.0008 | 0.112 ± 0.008 |
| Cellulose | 6.36 | 0.121 ± 0.0006 | 0.157 ± 0.001 | 5.46 | 0.131 ± 0.001 | 0.539 ± 0.0001 |
| Xylan | 6.3 | 0.235 ± 0.001 | 2.447 ± 0.006 | 5.7 | 0.158 ± 0.0003 | 1.656 ± 0.01 |
| Xylose | 6.5 | 0.021 ± 0.0007 | 1.223 ± 0.006 | 4.6 | 0.055 ± 0.0008 | 1.072 ± 0.009 |
| Glucose | 6.36 | 0.016 ± 0.0009 | 1.631 ± 0.009 | 4.58 | 0.032 ± 0.001 | 1.480 ± 0.012 |
| Sucrose | 6.2 | 0.019 ± 0.0006 | 1.845 ± 0.007 | 5.2 | 0.044 ± 0.0005 | 1.518 ± 0.006 |
| Lactose | 6.3 | 0.011 ± 0.0003 | 0.985 ± 0.003 | 5.4 | 0.021 ± 0.0005 | 1.179 ± 0.0001 |

The best xylan concentration for high level of xylanase from *Bacillus coagulans* and *Bacillus licheniformis* was 0.2 % (2.44 U/ml and 1.16 U/ml respectively) and when the xylan concentration increased the activity of enzyme decline significantly (LSD at 0.05 Level). But the maximum growth occurred at 0.4 % xylan concentration in basal media for the both strains as shown in table 3.

Table (3): Effect of different concentration of xylan on xylanase production and growth on *Bacillus coagulans and Bacillus licheniformis*

| Xylan Conc. (mg/ml) | *Bacillus coagulans* | | | *Bacillus licheniformis* | | |
|---|---|---|---|---|---|---|
| | Final pH | Optical density (OD600nm) | Xylanase activity (U/ml) | Final pH | Optical density (OD600nm) | Xylanase activity (U/ml) |
| 0.2% | 6.33 | 0.231 ± 0.0009 | 2.447 ± 0.006 | 5.69 | 0.143 ± 0.007 | 1.161 ± 0.003 |
| 0.4% | 6.3 | 0.544 ± 0.0003 | 1.832 ± 0.02 | 5.6 | 0.443 ± 0.0009 | 1.029 ± 0.003 |
| 0.6% | 6.21 | 0.535 ± 0.0009 | 1.675 ± 0.01 | 5 | 0.362 ± 0.002 | 0.797 ± 0.008 |
| 0.8% | 6.13 | 0.524 ± 0.0009 | 1.267 ± 0.005 | 4.8 | 0.257 ± 0.0009 | 0.64 ± 0.007 |
| 1.0% | 6 | 0.405 ± 0.0006 | 0.998 ± 0.008 | 4.3 | 0.162 ± 0.0009 | 0.201 ± 0.0003 |
| 1.2% | 5.98 | 0.396 ± 0.0009 | 0.232 ± 0.004 | 4.2 | 0.132 ± 0.0009 | 0.138 ± 0.006 |

An optimum temperature for xylanase production from *Bacillus coagulans* and *Bacillus licheniformis* was 50$^{o}$C as shown in fig.3 and fig.4. At 55$^{o}$C the activity was declined significantly (LSD at 0.05 Level).Maximum growth was attained at 50$^{o}$C.

The optimum pH for xylanase production from *Bacillus coagulans* was 6 with activity 2.9 U/ml

(fig.5) but from *Bacillus licheniformis* was 7 with activity 1.69 U/ml (fig.6). The enzyme activity decreased significantly (LSD at 0.05 Level) at pH 9 for *Bacillus coagulans* and at pH 8 for *Bacillus licheniformis.*

In contrast the best PH for growth for both strains was pH 3 and the growth declined extremely significantly (LSD at 0.05 Level) at pH 4.

**Physiochemical properties of the crude xylanase**

The activity of crude enzyme of *Bacillus coagulans* increased with increasing pH within pH range of 3 to 6 until reached the optimum value of pH 6 (fig.7), but for *Bacillus licheniformis*, the activity of crude enzyme increased with increasing pH within pH range of 3 to 8 until reached the optimum value of pH 8 (fig.7). The relative activity decreased extremely significantly (LSD at 0.05 Level) and reached minimum levels at pH 10 for both strains. The activity of crude xylanase increased with increasing temperature starting from 30°C till reached the optimum at 50°C for *Bacillus coagulans* and 70°C for *Bacillus licheniformis* (fig.8). The minimum level of activity of the crude enzyme for both strains reached significantly (LSD at 0.05 Level) at 80°C.

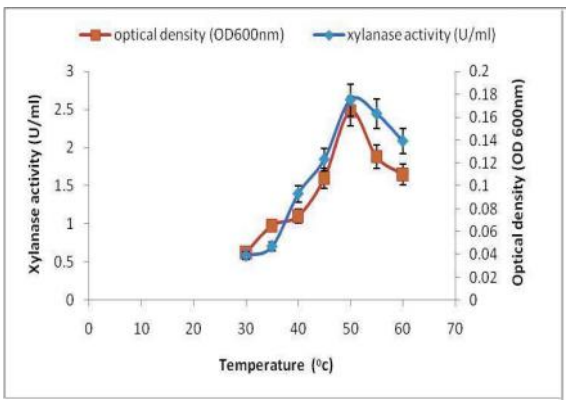


Fig (3): Effect of different temperatures on xylanase production and growth of *Bacillus coagulans*.

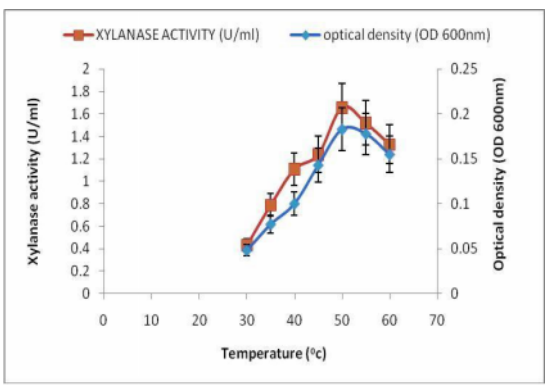


Fig (4): Effect of different temperatures on xylanase production and growth of *Bacillus licheniformis*.

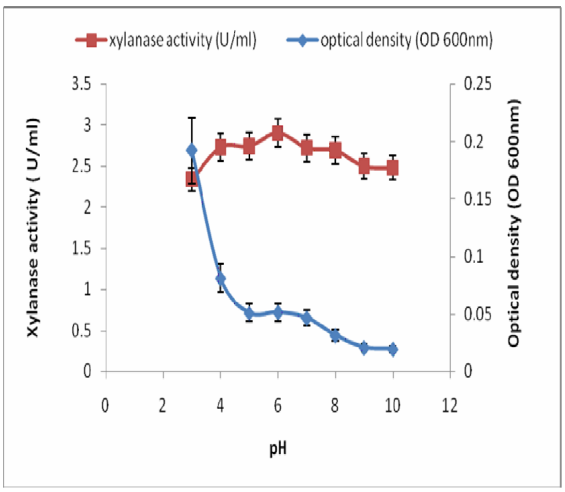


Fig (5): Effect of different pH on xylanase production and growth of *Bacillus coagulans*.

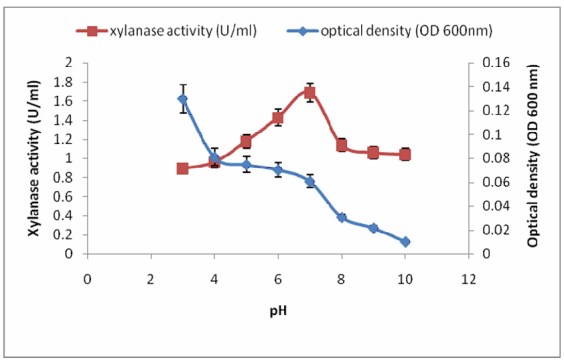


Fig (6): Effect of different pH on xylanase production and growth of *Bacillus licheniformis.*

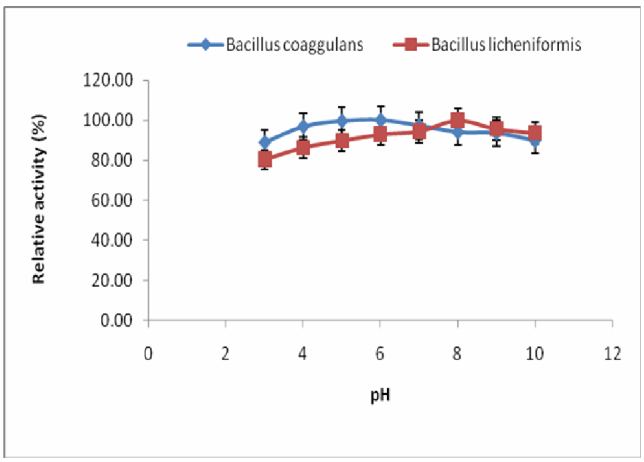


Fig (7): Effect of pH on activity of crude xylanase of *Bacillus coagulans* and *Bacillus licheniformis*

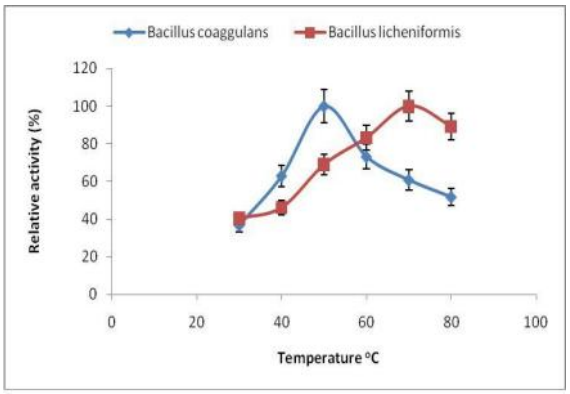


Fig (8): Effect of temperature on activity of crude xylanase of *Bacillus coagulans* and *Bacillus licheniformis*

The Km and Vmax values for crude enzyme for both strains are listed in table (4).

Table (4): Km and Vmax values of crude xylanase of *Bacillus coagulans* and *Bacillus licheniformis*

| Values | *Bacillus coagulans* | *Bacillus licheniformis* |
|---|---|---|
| Km (mg/ml) | 3.0 | 4.0 |
| Vmax (µmole/ml/min.) | 0.641 | 0.568 |

The thermal stability for crude enzyme from *Bacillus coagulans* was completely stable at 60°C, 70°C and 80°C for 5 hours and at 50°C for 4 hours (fig.9). The crude enzyme lost 50% of its activity with extremely significant (LSD at 0.05 Level) in (6 hours and 42 minutes) and (6 hours and 54 minutes) at 50°C and 60°C respectively, but at 70°C and 80°C the half life time of xylanase enzyme at was 7 hours. But the thermal stability for crude enzyme from *Bacillus licheniformis* was completely stable at 60°C, 70°C and 80°C for 6 hrs and at 50°C for 5 hrs (fig.10). The crude enzyme lost 50% of its activity with extremely significant (LSD at 0.05 Level) in 7 hours, (7 hours and 12 minutes) and (7 hours and 42 minutes) at 50°C, 60°C and 70°C respectively, but at 80°C the half life time of xylanase was 8 hours.

**4. Discussion**

Growth and xylanase production by *Bacillus coagulans* and *Bacillus licheniformis* increased reaching maximum values after 48 hrs and 60 hrs respectively, after which, the production of the enzyme decreased. The observed peaking and thronging of the production of extracellular enzymes can be attributed to (1) the products of action of one component inducing the synthesis of another, (2) differential inhibition by products of substrate hydrolysis, (3) decreased in growth observed after 48 hrs of growth of *Bacillus coagulans* and 60 hrs of growth of *Bacillus licheniformis*. This probably resulted from cellular lysis, an observation previously reported. These results indicate that the production of extracellular xylanase by *Bacillus coagulans* and *Bacillus licheniformis* was growth associated and this is in agreement with other investigators (Bajpai and Bajpai, 1989; Stephenson et al., 1998; Riaz et al., 2003) which Xylanases produced by Bacillus sp. were growth-associated, reaching a maximum after 24 hrs, and the enzyme production remained more or less the same up to 48 hrs (Anuradha et al., 2007) and (Azeri et al., 2010). On the other hand, *Bacillus amyloliquefaciens* secreted the highest xylanase activity in the culture supernatant after 48 hrs of growth (Breccia et al., 1998).

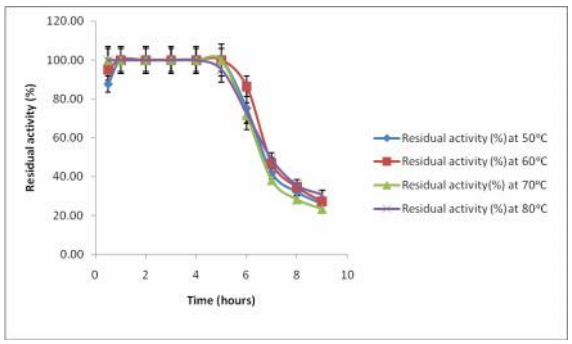


Fig (9): Thermal stability of xylanase of *Bacillus coagulans*

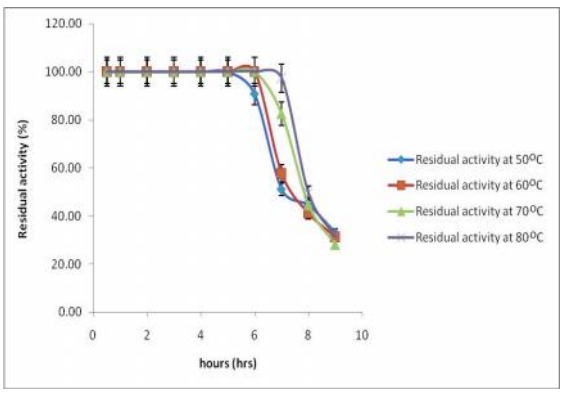


Fig (10): Thermal stability of xylanase of *Bacillus licheniformis*

The optimal production of xylanase and growth of *Bacillus coagulans* and *Bacillus licheniformis* could be obtained when ammonium sulfate and peptone were used as nitrogen sources respectively, ammonium sulfate is very good inorganic nitrogen source for producing xylanase enzyme in high activity and many researches agreement with this, for example many researchers on *Bacillus subtilis* agreement with this fact, Hoq et al., (1994) reported that ammonium phosphate or yeast extract, when

substituted for ammonium sulphate or peptone, showed appositive effect on biomass, soluble protein and xylanase yield from *Thermomyces lanuginose* RT9. Also peptone is very good organic nitrogen source for producing xylanase in high activity. Similar results were reported by Battan et al., (2007). Highest xylanase production (251 IU/ml) by *Bacillus* SSP-34 occurred in a medium containing yeast extract and peptone each at 0.25% (Subramaniyan et al., 2001). However, the best nitrogen source for xylanase production by *B. circulans AB16* (Dhillon and Khanna, 2000) and *Geobacillus thermoleovorans* (Sharma et al., 2007) was tryptone. Peptone containing media contain high nutrional amino acids and this may lead to high xylanase production and good growth.

Carbon source is one of the essential constituents of the microbial fermentation medium, which affects the overall cellular growth and metabolism (Nagar et al., 2010). The best carbon source for xylanase production from *Bacillus coagulans* and *Bacillus licheniformis* was found to be xylan. Xylan is know to induce xylanase production in different bacterial strains. The activity of xylanase is much lower in the presence of sugars compared with xylan, similar observation were recorded by Nagar et al. (2010). Glucose showed repression effect on enzyme production. It has been reported that the synthesis of carbohydrate degrading enzymes in most species is subjected to catabolite repression by readily metabolite substance such as glucose.

Xyanase activity in presence of carboxymethylcellulose and cellulose is very low and in presence of starch,no xylanase production from *Bacillus coagulans* and may be that is because these substrates make inhibition to xylanase production, and similar observations were recorded by Nagar et al., (2010). Xylanase production was found to vary with change in the concentration of xylan. Enzyme activity was measured in the presence of 0.2–1.2% xylan. It was found to be highest with 0.2% (w/v) xylan, and there was a decline in xylanase production on increasing the concentration of xylan beyond 0.2%. This could be due to formation of a thick suspension and improper mixing of the substrates in shake flasks, and similar observations were recorded by Nagar et al. (2010).

The optimum temperature for production of xylanase and growth of *Bacillus coagulans* and *Bacillus licheniformis* was 50$^{o}$C.These results are similar to other experiments studied alkaline tolerant xylanase production by *Bacillus subtilis* isolated from marine environment (Annamalai, 2009) and also similar to other results studied cellulose-free xylanase from thermoalkalophilic *Bacillus* spp JB99 (Kumar et al., 2011). xylanase Reported by other worker has optimum temperature at 60$^{o}$C (Azeri et al., 2010) but in this study the optimum temperature was 50$^{o}$C which indicate the possible industrial use of this enzyme (Annamalai, 2009) because Heat stability in xylanases is essentially required for their application in pulp and paper processing (Chetna Joshia and S.K. Khare, 2011).Also these results agree with studies on xylanase production by *Bacillus pumilus* SV-85S (Nagar et al., 2010),

Bacterial cells have various mechanisms that allow them strictly to control excretion (Mamo and Gessesse, 1999). Change in the natural of cell envelope can affect the realease of extracellular enzymes to the cultural medium (Antranikian, 1990). Temperature is the one of the factors that induces such changes on the cell membranes and cell walls (De vrij et al., 1990; Nordstrom, 1993). It is also reported that in Bacillaceae , the surface protein layer (S-layer) is involved in the control of exoenzyme release (Egelseer et al., 1996).

Extracellular xylanase production of *Bacillus coagulans* and *Bacillus licheniformis* was optimum at pH 6 and pH 7 respectively. In other hand, both organisms gave high growth at pH 3, and this is indicates that these two strains prefer to grow in acidic medium but optimum pH for giving high activity of xylanase is between pH 6 and pH 7, and these results are similar to the observations that were recorded by studies on xylanase production by *Bacillus subtilis* (Nagar et al., 2010).

Also Similar pH optimum for xylanase production was reported by Kohli et al. (2001). However, enzyme production by *B. subtilis* ASH (Sanghi et al., 2009), *B. circulans* AB 16 (Dhillon and Khanna, 2000), *B. pumilus* ASH (Battan et al., 2007), and *Bacillus licheniformis* (Archana and Satyanarayana, 1998) was highest at pH 7.0. In other hand there are results studied cellulose-free xylanase from thermoalkalophilic *Bacillus* spp JB99 showed optimum pH 10 (Kumar et al., 2011). The composition of cell wall and plasma membarane of microorganisms is known to be affected by the culture pH (Ellwood and Tempeat, 1972a & b). The change of the medium may lead to change of the nature of the cell membrane and/or cell wall and hence affecting the xylanase production and the growth of *Bacillus coagulans* and *Bacillus licheniformis.*

Most known xylanases so far have their optimum pH around neutrality. The pH optimal for the crude xylanase of *Bacillus coagulans* and *Bacillus licheniformis* was found to occur at pH 6 and pH 8 respectively. These results are in accordance with the observations that were recorded by studies on xylanase production by *Bacillus subtilis* (Nagar et al., 2010). Also this is in agreement with the pH optima of xylanase from alkalophilic

thermophilic *Bacillus sp*. (Bataillon et al., 1998).

The crude xylanase of *Bacillus coagulans* showed temperature optima at 50°C and in other hand the crude xylanase of *Bacillus licheniformis* showed temperature optima at 70°C, and these results are in accordance with the observations that were recorded by studies on xylanase production by *Bacillus subtilis* (Nagar et al., 2010). An identical temperature optimum was reported for the enzyme from *Bacillus sp*. (Blanco et al., 1995; Dey et al., 1992; Nakamura., 1993; Poorna and Prema, 2006). Only a few xylanases are reported active and stable at both alkaline pH and elevated temperature (Nakamura et al., 1993; Subramaniyan, 2001) and the xylanase production by *Bacillus licheniformis* in this study from this type.

The relative activity of crude xylanase production by *Bacillus coagulans* was completely stable at 60°C, 70°C and 80°C for 5 hrs and at 50°C for 4 hrs. The crude enzyme lost 50 % of its activity in (6 hours and 42 minutes) and (6 hours and 54 minutes) at 50°C and 60°C respectively, but at 70°C and 80°C the half life time of xylanase enzyme at was 7 hours. And the thermal stability for crude enzyme from *Bacillus licheniformis* was completely stable at 60°C, 70°C and 80°C for 6 hrs but at 50°C for 5 hrs. The crude enzyme lost 50% of its activity in 7 hours, (7 hours and 12 minutes) and (7 hours and 42 minutes) at 50°C, 60°C and 70°C respectively, but at 80°C the half life time of xylanase was 8 hours.

And that is indicate that the high temperature make activation to xylanase production by the two strains, so the xylanases from *Bacillus coagulans* and *Bacillus licheniformis* are thermostabe xylanases, similar to the thermostable xylanases already recorded from *Bacillus* sp. (Annamalai, 2009).

Also these results are similar to studies on xylanase from *Bacillus stearothermophilus T-6* which exposure to 65°C for more than 10 hrs did not effect the activity, and at 70 and 75°C, the half-lives of the enzyme were about 14.5 hrs and 20 min respectively. (Khasin et al., 1993). At pH 9.0 and 65°C, the half-life of the enzyme was about 6 hrs (Klibanov, 1983), and this phenomenon called thermoinactivation, suggests that thermoinactivation is controlled by a monomolecular conformational process (unfolding of the native protein) (Klibanov, 1983; Klibanov and Volkin, 1989).

The crude xylanase from *Bacillus coagulans* showed good affinity to xylan with Km and Vmax values of 3.0 mg/ml and 0.6410 µmole/min./ml, respectively. The affinity of xylanase enzyme towards xylan was similar to the xylanases isolated from other bacteria such as *Bacillus* sp NCIM 59 (Dey.D et al., 1992) and *Bacillus* sp 41M (S. Nakamura et al., 1993).In other hand the crude xylanase from *Bacillus licheniformis* showed less affinity to xylan with Km and Vmax values of 4.0 mg/ml and 0.5681 µmole/min./ml respectively, and these results similar to xylanase isolated from *Staphylococcus* sp (S. Gupta et al., 2000).

**Conclusion:**

The aims of this study are for producing xylanase with high activity from some bacteria isolated from soil of Egypt and study the factors affecting on the production process. The work also included studying the characterization of the crude enzyme.

So the optimum condition for the production of xylanase by *Bacillus coagulans* and *Bacillus licheniformis* can be summarized as follow:
48 hours and 60 hours incubation for *Bacillus coagulans* and *Bacillus licheniformis* respectively.

The pH 6.0 and pH 7.0 are the best pHs for the production of xylanase from *Bacillus coagulans* and *Bacillus licheniformis* respectively.

Temperature 50°C is the best temperatures for the production of xylanase from *Bacillus coagulans* and *Bacillus licheniformis.*

Oat splet xylan at concentration 0.2% is the best carbon source and best concentration for the production of xylanase from *Bacillus coagulans* and *Bacillus licheniformis.*

Ammonium sulphate and peptone are the best nitrogen sources for the production of xylanase from *Bacillus coagulans* and *Bacillus licheniformis* respectively.

Crude extracellular xylanase from *Bacillus coagulans* and *Bacillus licheniformis* grown in Sorenson medium was characterized. The optimum temperature and pH were found to be 50°C and pH 6.0 respectively for xylanase from *Bacillus coagulans* and 70°C and pH 8.0 respectively for xylanase from *Bacillus licheniformis.*

All of these criteria of xylanase from *Bacillus coagulans* and *Bacillus licheniformis* are very good criteria for production of industrial xylanase.

Before using xylanase at industrial level, several criteria have to be fulfilled. Pilot scale processes are generally carried out at high temperature; therefore bacterial xylanase having broad ranges of pH and temperature stability are preferred in industry. Similarly xylanase extracted from *Bacillus coagulans* and *Bacillus licheniformis*.

**Corresponding author**
**Reham A. El Fayoumy**
Botany Department (Microbiology), Faculty of Science, Mansoura University (Damietta Branch), New Damietta, Egypt**.**
reham85@mans.edu.eg

**Reference:**


1. Alexander Khasin, Iris Alchanati, & Yuval Shoham. (1993). Purification and characterization of athermostable xylanase from *Bacillus stearothermophilus* T-6. Applied and Environmental Microbiology. American Society for Microbiology 59; 6
2. Angelo, R., C. Aguirre, E. Curotto, E. Esposito, J.D. Fontana, M. Baron, A.M.F. Milagres & N. Durán. (1997). Stability and chemical modification of xylanase from *Aspergillus* sp (2MI strain). Biotechnol. Appl. Biochem., 25; 19-27.
3. Anuradha P, Vijayalakshmi K, Prasanna ND, & Sridevi K. (2007). Production and properties of alkaline xylanases from *Bacillus* sp. isolated from sugarcane fields. Curr Sci 90(9); 1283–1286
4. Anuradha, P., K. Vijayalakshmi, N.D. Prasanna, & K. Sridevi. (2007). Production and properties of alkaline xylanases from *Bacillus sp*. Isolated from
5. sugarcane fields. Curr.sci. 92; 1283-1286.
6. Archana A, Satyanarayana T. (1998). Cellulase-free xylanase production by thermophilic *Bacillus licheniformis* A99. Indian J Microbiol. 38;135–139
7. Bailey, M.J., J. Buchert and L. Viikari.(1993). Effect of pH on production of xylanase by *Trichoderma reesei* on xylan and cellulose based media. Appl. Microbiol. Biotechnol. 40; 224-229.
8. Bajpal, P., P. Bajpai. (1989). High temperature alkaline α-amylase from *Bacillus licheniformis* TCRFC-B13. Biotechnology and Bioengineering. 33; 72-78.
9. Battan B, Sharma J, Dhiman SS, Kuhad RC (2007). Enhanced production of cellulase-free thermostable by *Bacillus pumilus* ASH and its potential application in paper industry. Enzyme Microb Technol. 41; 733–739
10. Bataillon M, Nunes-Cardinali AP, Duchiron F (1998). Production of xylanase from newly isolated alkalophilic thermophilic *Bacillus* sp. Biotechnol Lett. 20;1067–1071
11. Blanco A, Vidal T, Colom JF, Pastor FIJ (1995). Purification and properties of xylanase A from alkali-tolerant *Bacillus* sp. Strain BP-23. Appl Env Microbiol. 61; 4468–4470
12. Breccia JD, Sineniz F, Baigori MD, Castro GR, Hatti KR (1998). Purification and characterization of a thermostable xylanase from *Bacillus amyloliquefaciens*. Enzyme Microb Technol. 22; 42–49
13. Cem Azeri, Abdurrahman U. Tamer & Mustafa Oskay. (2010). Thermoactive cellulase-free xylanase production from alkaliphilic *Bacillus* strains using various agro-residues and their potential in biobleaching of kraft pulp. African Journal of Biotechnology. 9 (1); 063-072.
14. Chetna Joshia and S.K. Khare. (2011). Utilization of deoiled Jatropha curcas seed cake for production of xylanase from thermophilic Scytalidium thermophilum. Bioresource Technology. 102; 1722-1726
15. Dey D, Hinge J, Shendye A, Rao M (1992). Purification and properties of extracellular endo-xylanase from alkalophilic thermophilic *Bacillus* sp. Can J Microbiol. 38; 436–442
16. Dhillon A, Khanna S (2000). Production of a thermostable alkalitolerant xylanase from *Bacillus circulans* AB 16 grown on wheat straw. World J Microbiol Biotechnol. 16; 325–327
17. El-Helow, E.R. and A. El-Ahawany, (1999). Lichenase production by catabolite repression resistant *Bacillus subtilis* mutants: Optimization
18. and formulation of an agro-industrial by-product medium. Enzyme Microbiol. Technol. 24; 325-331.
19. G.L. Miller, (1959). Use of dinitrosalycylic acid reagent for determination of reducing sugar, Anal. Chem. 31; 538-542.
20. Gilbert, M., M. Yaguchi, D.C. Watson, K.K.Y. Wong, C. Breuil & N. Saddler, (1993). A comparison of two xylanases from the thermophilic fungi *Thielavia terrestris* and *Thermoascus custaceus*. Appl. Micbiol. Biotechnol. 40; 508-514.
21. H. Lineweaver, (1934). D. Bruk, The determination of enzyme dissociation constants, J. Am. Chem. Soc. 56; 658-666.
22. Haltrich, D., M. Preiss and W. Steiner. (1993). Optimization of a culture medium for increased xylanase production by a wild strain of *Schizophyllum commune.* Enzyme Microbiol. Technol. 15; 854-860.
23. Haltrich, D., B. Laussamayer and W Steiner. (1994). Xylanase formation by *Sclerotium rolfsii:* Effect of growth substrates and development of a culture medium using statistically designed experiments. Appl. Micobiol. Biotechnol.,42; 522-530.
24. Hoq, M.M., Hempel, C. & Decker, W.D. (1994). Cellulose free xylanase by *Thermomyces lanuginosus* RT9: Effect of agitation, aeration and medium components on production. *J. Biotechnol*. 37; 49-58
25. Kar, S., A Mandal, P. Das Mohapatra, K. Mondal & K. Pati, (2006). Production of cellulose–free xylanase by *Trichoderma reesi* SAF3. Brazilian J. Microbiol. 37; 462-464.
26. Klibanov, A. M. (1983). Stabilization of enzymes against thermal inactivation. Adv. Appl. Microbiol. 29; 1-24.35.
27. Klibanov, A. M., and D. B. Volkin. (1989). Minimizing protein inactivation, p. 1-24. In T. E. Creighton (ed.), Protein function, a practical approach. IRL Press, Oxford.
28. Kulkarni, N., A. Shendye and M. Rao, (1999). Molecular and Biotechnological aspects of xylanases. FEMS. Microbiol. Rev. 23; 411-456.
29. Kohli U, Nigam P, Singh D, Chaudhary K (2001).

Thermostable, alkalophilic and cellulase free xylanase production by *Thermoactinomyces thalophilus* subgroup C. Enzyme Microb. Technol. 28; 606–610

30. Lynch, J.M., J.H Slater, J.A. Bennett & S.H.T. Harper, (1981). Cellulase activities of some aerobic microorganisms from soil. J. Gen. Microbiology. 127; 231-236.
31. Morales, P., A. Madarro, A. Flors, J.M. Sendra, J.A. Pérez-Gonzáles, (1995). Purification and vcharacterization of a xylanase and an arabinofuranosidade from *Bacillus polymyxa*. Enz. Microbial Technol. 17; 424-429.
32. N Annamalai, R Thavasi1, S Jayalakshmi & T Balasubramanian. (2009): Production and optimization of cellulase-free, alkali-stable xylanase by Bacillus pumilus SV-85S in submerged fermentation. Indian Journal of Biotechnology. 8; 291-297
33. Nakamura S, Wakabayashi K, Nakai R, Aono R, Horikoshi K (1993). Purification and some properties of an alkaline xylanase from alkaliphilic Bacillus sp. Strain 41M–1. Appl Environ Microbiol 59(7); 2311–2316
34. Prade, R.A., 1996. Xylanases from Biology to biotechnology. Biotechnol. Genet. Eng. Rev. 13; 101-131.
35. Poorna CA, Prema P (2006). Production and partial characterization of endoxylanase by *Bacillus pumilus* using agro industrial residues. Biochem Eng J. 32; 106–112.
36. Riaz, N., I. Ul-aq, M.A Qadeer. (2003). Characterization of amylase by Bacillus subtilis. International Journal of Agricultural Biology. 5(3); 249-252.
37. Sanghi A, Garg N, Kuhar K, Kuhad RC, Gupta VK (2009). Enhanced production of cellulase-free xylanase by alkalophilic Bacillus subtilis ASH and its application in biobleaching of kraft pulp. BioResources. 4(3); 1109–1129
38. Sneath, P. H., & M. STEVENS, (1985). Anumerical taxonomic study of *Actinobacillus*, *Pasteurella* and *Yersinia*. J. Gen. Microbiol. 131; 2711–2738.
39. S. Nakamura, K. Wakabayashi, R. Nakai, R. Aono, K. Horikoshi, (1993). Purification and some properties of an alkaline xylanase from an alkaliphilic *Bacillus* sp Strain 41M-1, world J. Microbiol Biotechnol. 59; 2311-2316.
40. Shashi shekhar kumar1, D. D. Panday2 and G.R. Naik, (2011). Purification and molecular characterization of low molecular weight cellulose-free xylanase from thermoalkalophilic *Bacillus* spp.JB 99.world journal of science and technolog.1(2); 9-16
41. Shoham, Y., Z. Schwartz, A. Khashin, O. Gat, Z. Zosim & E. Rosenberg, (1992). Delignification of wood pulp by a thermostable xylanase from *Bacillus stearothermophilus* T-6. Biodegradation. 3; 207-218.
42. Sharma A, Adahikari S, Styanarayana T (2007). Alkali-thermostable and cellulase-free xylanase production by an extreme thermophile Geobacillus thermoleovorans. World J Microbiol Biotechnol. 23; 483–490
43. Stephenson, K., N.Mo. Carter, C.R Harwood, M.F. Petitglatron, R. Chambert. (1998). The influence of protein folding on late stages of the secretion of α-amilase from Bacillus subtilis. FEBS Letter 430; 385-389.
44. Subramaniyan, S. and P.Prema, (2000). Cellulose free xylanases from *Bacillus* and other microorganisms. FEMS. Lett. Microbiol. 183; 1-7.
45. Subramaniyan S, Sandhia GS, Prema P (2001). Biotech control of xylanase production without protease activity in Bacillus sp. by selection of nitrogen source. Biotechnol Lett 23:369–371
46. Sushil Nagar, Vijay Kumar Gupta, Davender Kumar, Lalit Kumar, Ramesh Chander Kuhad. (2010). Production and optimization of cellulase-free, alkali-stable xylanase by Bacillus pumilus SV-85S in submerged fermentation. J Ind Microbiol Biotechnol. 37; 71–83
47. Subramaniyan S, Sandhia GS, Prema P (2001). Biotech control of xylanase production without protease activity in Bacillus sp. by selection of nitrogen source. Biotechnol Lett. 23; 369–371
48. S. Gupta, B.Bhushan, G.S. Hoondal, (2000). Isolation, purification and characterization of xylanase from *Staphylococcus* sp SG-13 and its application in biobleaching of craft pulp. J. Appl. Microbiol. 88; 325-334.
49. Yang, S.Q., Q.J. Yan, Z.Q. Jiang, T. Lil, H.M. Tian & Y.Z. Wang, (2006). High level of xylanase production by the thermophilic *Paecilomyces themophila* J18 on wheat straw in solid state fermentation. Bioresource. Technol. 97; 1794-1800.

11/21/2011