Kinetics and Thermodynamics of Thermal Inactivation of Cellulase from Salivary Glands of *Macrotermes subhyalinus* Little Soldier

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Abstract: For optimization of biochemical processes in food and pharmaceutical industries, the evaluation of enzyme inactivation kinetic models is necessary to allow their adequate use. kinetics and thermodynamic analysis of cellulase (GS-CX) from salivary glands of *Macrotermes subhyalinus* little soldier were studied, using carboxymethylcellulose as a substrate. Optimal conditions for enzymatic studies were determined to be pH 5.0 and 60 °C. Thermal inactivation of GS-CX was examined in more detail between 50 and 65 °C and in relation to exposure time. The investigation suggests the existence of a non-sensitive heat fraction on the enzyme structure, which is relatively stable up to temperatures close to 55 °C. Denaturation of this enzyme, measured by loss in activity, could be described as a first-order model, with k-values between 0.0052 and 0.0337 min⁻¹. D- and k-values decreased and increased, respectively, with increasing temperature, indicating faster cellulase (GS-CX) inactivation at higher temperatures. Results suggested that GS-CX is a relatively thermostable enzyme with a z-value of 18.08 °C and Ea of 115.81 kJmol⁻¹. The results of the thermodynamic investigations indicated that the hydrolytic reactions were: (1) not spontaneous (ΔG > 0) and (2) slightly endothermic (ΔH > 0). Positive values of entropy (ΔS > 0) for GS-CX indicated that this enzyme is found in a chaotic state at the end of the reaction. The high value obtained for the variation in enthalpy indicated that a high amount of energy was required to initiate denaturation, probably due to the molecular conformation of this enzymes. Results shown that the enzyme is quite stable for biotechnological applications.

Keywords: Carboxymethylcellulose, Kinetics And Thermodynamic Parameters, *Macrotermes Subhyalinus*, Thermal Stability, Salivary Glands, Cellulase

Introduction
The major nutritional component for wood-feeding termites is cellulose, which is plant’s main structural constituent. Apart from being structural pests, termites have a huge impact on terrestrial ecological processes, as they are part of the main terrestrial cellulose recyclers (Inoue et al., 1997). Cellulase activity in the midgut of these insects is very high (Hogan et al., 1988), and studies have shown that they rely solely on their own cellulases (Slaytor, 1992; Slaytor, 2000) rather than the cellulases of symbiotic bacteria present in their lower intestine (Brune and Stingl, 2005). In nature, cellulose degradation requires the synergistic action of three types of glycoside hydrolases (GH): endo-β-1,4-glucanases (EG; EC 3.2.1.4), exo-β-1, 4-cellobiohydrolases (CBH; EC. 3.2.1.91), and β-glucosidases (EC. 3.2.1.21) (Clarke, 1997). Cellulase is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds (Parry et al., 2001). Based on their mode of action, three varieties of cellulase exist which any highly cellulolytic organism must possess (Breznak and Brune 1994). Exoglucanases (EC 3.2.1.91) are the first class of cellulases. The second class of cellulases consists of the endoglucanases (EC 3.2.1.4) while the third class is the beta-glucosidases (EC 3.2.1.21). Cellulases contribute to 8% of the worldwide industrial enzyme demands (Elba and Maria, 2007). The cellulase market is expected to expand dramatically when cellulases are used to hydrolyzed pretreated cellulosic material to sugars, which can be fermented to bioethanol and biobased products on large scales. Thus, the use of cellulase in various industrial processes is indispensable. It is used in plants and agricultural waste processing (Mswaka and Magan, 1998; Lu et al., 2004), biofuels (Vaithanomsat et al., 2009), triphase biomethanation (Chakraborty et al., 2000), chiral separation and ligand binding studies (Nutt et al., 1998). In addition to this, the major industrial application 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 (Hill et al., 2006). Besides, they are used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juice and in baking, while de-inking of paper is yet another emerging application. A potential challenging area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals (Lynd et al., 2005). Based on the importance of lignocellulose-degrading enzymes, it is necessary to know optimal conditions of enzyme active sites functioning and the effect of different physical-chemical factors on their activity. Hus, any process that enhances the structural stability and reaction rate of enzymes has a favorable impact on their industrial application (Sousa, 1995; Matsumoto et al., 1997). However, there is no report concerning thermal stability of these cellulases. Thus, the knowledge on kinetics of thermal inactivation of cellulase GS-CX is important to allow their suitable utilization as biocatalysts in industry. The present study focus on the investigation of kinetic and thermodynamic parameters of cellulase (GS-CX) from salivary glands of Macrotermes subhyalinus little.

Materials and Methods

Enzyme sources

Little soldiers of the termite Macrotermes subhyalinus were from the savannah of Lamto (Abidjan, Côte d’Ivoire). They were collected directly from their nests and then stored frozen at -20°C. The cellulase substrate carboxymethylcellulose was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All other chemicals and reagents were of analytical grade.

Extraction and purification procedures

Salivary glands (10 g) were dissected and homogenized with 20 mL 0.9 % NaCl (w/v) solution using a blender (Ultra-Turrax) and then sonicated as previously described by Rouland et al. (1988a). The homogenate was centrifuged at 20,000 x g for 15 min. The collected supernatant constituted the crude extract. After freezing at -180°C in liquid nitrogen, the crude extract was stored at -20°C (Kouamé et al., 2005).

The purification procedure was carried out in the cold room (4°C). The crude extract of the termite salivary glands was loaded onto an anion-exchange chromatography using a DEAE-Sepharose Fast Flow column (2.5 cm x 4.5 cm), equilibrated with 20 mM sodium acetate buffer (pH 5.0), and fractions of 2 mL were collected. One peak of enzyme possessing endo-xylanase/endo-cellulase activity was obtained and the active fractions were pooled. The pooled active fractions were loaded onto a cation-exchange chromatography using a CM-Sepharose CL-6B column (2.6 cm x 4.0 cm), equilibrated with 20 mM sodium acetate buffer (pH 5.0). The column was washed with the same buffer at a flow rate of 90 mL/h. Cellulase activity was eluted with a stepwise salt gradient (0.1, 0.2, 0.4 and 2 M) of NaCl in 20 mM sodium acetate buffer (pH 5.0), and fractions of 2 mL were collected. Fractions of 2 mL were collected and, to the pooled active fractions, solid sodium thiosulphate was slowly added to give a final concentration of 1.7 M and the resulting enzyme solution was subsequently applied on a Phenyl Sepharose 6 Fast Flow column (1.5 cm x 3.2 cm) previously equilibrated with 20 mM sodium acetate buffer (pH 5.0) containing 1.7 M of sodium thiosulphate salt. The column was washed with a reverse stepwise gradient of sodium thiosulphate concentrations (from 1.7-0 M) dissolved in the same sodium acetate buffer at a flow rate of 78 mL/h and fractions of 1 mL were collected. The pooled active fractions were dialyzed overnight against 20 mM sodium acetate buffer (pH 5.0) and constituted the purified enzyme solution.

Enzyme assay and protein determination

Under the standard test conditions, cellulase activity was assayed spectrophotometrically by measuring the release of reducing sugars from Birchwood carboxymethylcellulose (CMC). The reaction mixture (0.38 ml) contained 0.2 ml of 0.5% CMC (w/v) dissolved in 20 mM acetate buffer (pH 5.0) and 0.1 ml enzyme solution. After 30 min of incubation at 45°C, the reaction was terminated by adding 0.3 ml of dinitrosaliclyc acid solution followed by 5 min incubation in a boiling water bath. The product was analysed by measuring the optical density at 540 nm. One unit (U) of enzyme activity was defined as the amount of enzyme capable of releasing one μmol of reducing sugar per min under the defined reaction conditions. Specific activity was expressed as units per mg of protein (U/mg of protein). Protein concentrations were determined spectrophotometrically at 660 nm by method of Lowry et al. (1951) using bovine serum albumin as a standard.

Thermal inactivation

Thermal inactivation of the enzyme was investigated at various constant temperatures from 50 to 65°C after exposure to each temperature for a period of 5 to 60 min. The enzyme was heated in sealed tubes, which was incubated in 100 mM sodium acetate buffer (pH 5.0) in a thermostatically controlled water
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bath. Tubes were withdrawn at each time intervals and immediately immersed in an ice bath, in order to stop heat inactivation. The residual enzymatic activity, determined in both cases at 37°C under the standard test conditions, was expressed as percentage activity of zero-time control of the untreated enzyme.

Kinetic data Analysis
First-order kinetic has been reported to describe thermal inactivation of cellulase (GS-CX) (Guiavarch et al., 2002). The integral effect of inactivation process at constant temperature, where the inactivation rate constant is independent of time, is given in Eq. 1:

\[ \ln \left( \frac{A_t}{A_0} \right) = -kt \quad \text{(Eq. 1)} \]

where:
- \(A_t\) is the residual enzyme activity at time \(t\) (min),
- \(A_0\) is the initial enzyme activity,
- \(k\) (min\(^{-1}\)) is the inactivation rate constant at a given condition.

\(k\)-values were obtained from the regression line of ln (\(A_t/A_0\)) versus time as slope.

D-value is defined as the time needed, at a constant temperature, to reduce the initial enzyme activity (\(A_0\)) by 90 %.

For first-order reactions, the D-value is directly related to the rate constant \(k\) (Eq. 2) (Stumbo, 1973; Espachs-Barroso et al., 2006):

\[ D = \frac{2.303}{k} \quad \text{(Eq. 2)} \]

The Z-value (°C) is the temperature increase needed to induce a 10-fold reduction in D-value (Stumbo, 1973). This Z-value follows the Eq. 3:

\[ \log \left( \frac{D_2}{D_1} \right) = \frac{T_2 - T_1}{Z} \quad \text{(Eq. 3)} \]

where,
- \(T_1\) and \(T_2\) are the lower and higher temperatures in °C or K.
Then, \(D_1\) and \(D_2\) are D-values at the lower and higher temperatures in min, respectively.

The Z-values were determined from the linear regression of log (D) and temperature (T).

Thermodynamic Analysis
The Arrhenius equation is usually utilized to describe the temperature effect on the inactivation rate constants and the dependence is given by (Eq. 4 or 5):

\[ k = A e^{-\frac{Ea}{RT}} \quad \text{(Eq. 4)} \]

Eq. 4 can be transformed to: \( \ln k = \ln A - \frac{Ea}{R} \times T \) (Eq. 5)

where:
- \(k\) is the reaction rate constant value,
- \(A\) the Arrhenius constant,
- \(Ea\) (kJ mol\(^{-1}\)) the activation energy,
- \(R\) (8.31 J mol\(^{-1}\) K\(^{-1}\)) the universal gas constant
- \(T\) is the absolute temperature in Kelvin (K).

When the “ln” of “k” is plotted against the reciprocal of the absolute temperature, a linear relationship should be observed in the temperature range studied.

The slope of the line obtained permitted to calculate the activation energy and the ordinate intercept corresponds to ln \(A\) (Dogan et al., 2000 and 2002).

The values of the activation energy (\(Ea\)) and Arrhenius constant (\(A\)) allowed the determination of different thermodynamic parameters (Marin et al., 2003) such as variations in enthalpy, entropy and Gibbs free energy, \(\Delta H\), \(\Delta S\) and \(\Delta G\), respectively, according to the following expressions (Galani and Owusu, 1997):

\[ \Delta H^\circ = Ea - RT \quad \text{(Eq. 6)} \]

\[ \Delta S^\circ = R (\ln A - \ln K_P h_p - \ln T) \quad \text{(Eq. 7)} \]

\[ \Delta G^\circ = \Delta H^\circ - T \Delta S^\circ \quad \text{(Eq. 8)} \]

Where:
- \(K_P\) is the Boltzmann constant (1.38 x 10\(^{-23}\) J/K),
- \(h_p\) is the Planck constant (6.626 x 10\(^{-34}\) J.s),
- \(T\) is the absolute temperature.

Statistical Analyses
All determinations reported in this study were carried out in triplicate. Results were expressed as means ± standard deviation.

Results and Discussion
Kinetic Analysis of Thermal Denaturation

The optimum temperature of cellulase (GS-CX) purified from salivary glands of Macrotermes subhyalinus little soldier was 60 °C (Fagohoun et al., 2014). In this study, thermal inactivation of Macrotermes subhyalinus little soldier GS-CX was screened at different temperatures ranged from 50 to 65 °C by determining the residual enzymatic activity (Table 1). The cellulase (GS-CX) activity was decreased with increasing heating time (5 - 60 min) and temperature (50 – 65 °C). Indeed, at temperatures between 50 and 65 °C, heat-denaturation of cellulase (GS-CX) occurred after 5 min of incubation (97.73 to 85.21 %). Although heating at 60°C for 30 min resulted in partial (50.76 %) inactivation. However, a strong inactivation of both enzyme activities was obtained after 60 min of heat treatment at 65°C (13.53 %). On one hand, the decrease of percentage residual activity at temperatures higher than 50 °C was most likely due to the unfolding of the tertiary structure of the enzyme to form the secondary structure and on other hand, it could be explained by the chemical modification (Tabatabai 1982). It has been noted that heat stability of the enzyme may be related to molecular forms of enzyme (Zhou and Feng 1991).

The logarithmic linear relationship between the cellulase activity and heat treatment time for the temperature ranged from 50 to 65 °C followed first-order kinetics (Figure 1). This result agrees with those reported for cellulase from fungi trichoderma.

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viride (Abdul, 2015) and in rice Grasshopper *hieroglyphus banian* (Haloi *et al*., 2012)

**Table 1:** Effect of treatment temperature and time on the inactivation of cellulase from salivary glands of *Macrotermes subhyalinus* little soldier.

| Temperature (°C) | 5° | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 |
|-----------------|----|----|----|----|----|----|----|----|----|----|----|----|
| 50              | 97.73± 0.8 | 95.12± 0.9 | 93.24± 1.0 | 86.07± 1.1 | 87.80± 0.9 | 86.07± 1.0 | 83.52± 0.9 | 80.65± 0.9 | 78.89± 0.9 | 77.88± 1.0 | 74.82± 0.9 | 73.34± 1.0 |
| 55              | 95.12± 1.0 | 91.39± 0.9 | 86.07± 0.9 | 81.06± 0.9 | 76.33± 0.9 | 76.33± 0.9 | 69.07± 0.9 | 66.36± 0.9 | 63.76± 0.9 | 60.04± 0.9 | 57.69± 0.9 | 54.33± 0.9 |
| 60              | 88.69± 1.0 | 79.45± 0.9 | 69.07± 0.9 | 63.76± 0.9 | 55.98± 0.9 | 50.76± 0.9 | 44.61± 0.9 | 43.07± 0.9 | 38.46± 0.9 | 33.62± 0.9 | 29.81± 0.9 | 27.25± 0.9 |
| 65              | 85.21± 1.0 | 74.08± 0.9 | 61.26± 0.9 | 49.65± 0.9 | 44.93± 0.9 | 36.78± 0.9 | 31.66± 0.9 | 25.66± 0.9 | 21.22± 0.9 | 18.64± 0.9 | 14.95± 0.9 | 13.53± 0.9 |

**Figure 1:** Thermal inactivation curves of cellulase (GS-CX) from salivary glands of *Macrotermes subhyalinus* little soldier in sodium acetate buffer (pH 5.0) in the temperature range 50-65 °C. A₀ is the initial enzymatic activity and Aᵣ the activity at each holding time. Each data point is the mean of three determinations.

From the slopes of these lines, the inactivation rate constants (k) were calculated and are given in Table 2. The rate constant increased with the heating temperature, indicating that cellulase (GS-CX) from salivary glands of *Macrotermes subhyalinus* little soldier is less thermostable at higher temperatures (60-65 °C). The dependence of the k-values with temperature was adequately fitted by the Arrhenius equation (R² = 0.990) (Figure 2). This linearity is an indication that the inactivation in salivary glands GS-CX occurs through a unique mechanism dependent on temperature, such as protein unfolding (Gnangui *et al*., 2009; Waliszewski *et al*., 2009).

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![Figure 2: Arrhenius plot showing the effect of temperature on the rate constant for the thermal inactivation of cellulase (GS-CX) from salivary glands of *Macrotermes subhyalinus* little soldier. 1/T represents the reciprocal of the absolute temperature. Each data point is the mean of three determinations](http://www.ijSciences.com)

The half-life ($t_{1/2}$) and the decimal reduction time (D-value) are other important parameters commonly used in the characterization of enzyme stability. Increasing the temperature from 50 to 65 °C resulted in a decrease in $t_{1/2}$ and D-values (Table 2). At the same temperature (65 °C), cellulase (GS-CX) from salivary glands of *Macrotermes subhyalinus* little soldier ($t_{1/2} = 20.56$ min) was less thermostable than those of other vegetal sources such as exo-PG from *Linhagem Fungica Thermomucor indicaseudaticae* (t$_{1/2}$ = 58.3 min) (Martin, 2010) and exo-polygalacturonase (exo-PG) from *Thermophilic Fungus Rhizomucor pusillus* (t$_{1/2}$ = 96.3 min) (Trindade et al., 2016).

From 50 to 65 °C, the activation energy (Ea) value for thermal inactivation of cellulase (GS-CX) was calculated to be 115.81 kJ.mol$^{-1}$ (Table 2). High activation energy reflects a greater sensitivity of cellulase to temperature change.

![Table 2: k, D-, t$_{1/2}$, Z- and Ea-values for thermal inactivation of cellulase from salivary glands of *Macrotermes subhyalinus* little soldier in the 50-65 °C temperature range.](http://www.ijSciences.com)

![Figure 3](http://www.ijSciences.com) presents the relationship between decimal reduction time and temperature, where the slope of the curve represents -1/Z$_T$. The estimated value of

Macrotermes subhyalinus little soldier cellulase was 18.08 °C ($R^2 = 0.9885$). The Z-values for cooking...
and nutrients degradation (25-45°C) are generally greater than microbial inactivation (7-12°C) (Awuah et al., 2007). In fact, differences between the D- and Z-values of enzyme and nutrients are exploited to optimize thermal processes and can be exploited also to maintain carboxymethylcellulase activity after treatment. This indicates that any change in temperature processing affects more intensely the stability of cellulase (GS-CX). In this study, D-, Z- and k-values indicate that carboxymethylcellulase is heat stable and then can be used in high temperature short time and low temperature long time industrial processes such as pasteurization, where values of 65°C for 3-5 min and 55°C for 30 min, respectively, are generally considered. In fact, the D- and Z-values of carboxymethylcellulase is exploited to optimize thermal processes and to preserve enzyme activity after treatment. According to Barrett et al. (1999), high Z-values indicate more sensitivity to the heat treatment time and low Z-values indicate more sensitivity to increasing temperature.

**Figure 3**: Variation of decimal reduction times with temperature for of cellulase (GS-CX) from salivary glands of Macrotermes subhyalinus little soldier. Each data point is the mean of three determinations

**Thermodynamic Analysis of Thermal Denaturation**

The thermodynamic parameters provide information on the enzyme thermal stability for each step of the heat-induced denaturation process. This could help in detecting any secondary stabilization or destabilization effects that would go unnoticed if only the half-life times were considered (Longo and Combes 1999). These parameters include the Gibbs free energy (ΔG*) change considered as the energy barrier for enzyme inactivation, the enthalpy (ΔH*) change measuring the number of bonds broken during inactivation, and the entropy (ΔS*) change that indicates the net enzyme and solvent disorder. In this study, they were calculated in the temperature range from 50 to 65 °C (Table 3).

Within the error range of our measurements, results showed that the enthalpy (ΔH*) is independent of temperature; thus, there is no change in enzyme heat capacity. The ΔH* value of cellulase (89.94 kJmol⁻¹) was slightly increased from 109.36 kJmol⁻¹ to 110.27 kJmol⁻¹, when the incubation temperature was elevated (50 - 65 °C) indicating that the inactivation processes were not spontaneous. Since ΔG* decreases with increasing temperature whereas ΔH* is overall constant, one could expect a significant contribution of entropy changes to the thermodynamics of the considered system. Indeed, it was already demonstrated that activation entropy has a dominant role in thermal inactivation of proteins in aqueous solutions (Bromberg et al., 2008).

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According to Anema and McKenna (1996), the positive values of entropy (ΔS#) for the hydrolysis reaction of carboxymethylcellulose indicate that the reaction proceeds with less speed and is characterized by low regularity. Small changes in the values of ΔS# indicates a preferential destruction of weak bonds (hydrogen and electrostatic), resulting in a lower loss of catalytic activity. The positive values for change in ΔS# also indicate that there are no significant processes of aggregation for carboxymethylcellulase.

Furthermore, the high values obtained for ΔS# variation probably reflect an increased disorder of the active site or the structure of cellulase, which is the main driving force of heat denaturation (D’Amico et al., 2003). The cellulase GS-CX from salivary glands of Macrotermes subhyalinus little soldier studied here therefore involves a structurally robust but temperature-sensitive enzymatic system, whose thermal denaturation is mainly under entropic control.

### Table 3: Thermodynamic parameters for the Thermal Inactivation of cellulase from salivary glands of Macrotermes subhyalinus little soldier at different Temperatures

| Temperature (°C) | ΔH# (kJ/mol) ± SD | ΔS# (J mol⁻¹ K⁻¹) ± SD | ΔG# (kJ/mol) ± SD |
|------------------|------------------|------------------------|------------------|
| 50               | 113.13 ± 0.6²    | 126.23 ± 1.6           | 72.33 ± 0.3      |
| 55               | 113.08 ± 0.5     | 126.10 ± 1.7           | 71.70 ± 0.4      |
| 60               | 113.04 ± 0.6     | 125.98 ± 0.3           | 71.07 ± 0.1      |
| 65               | 113.00 ± 0.4     | 125.85 ± 0.6           | 70.44 ± 0.1      |
| Mean             | 113.06 ± 0.5     | 126.04 ± 0.4           | 71.39 ± 0.3      |

a Mean (±SD) for triplicate experiments

### Conclusion

The results of this study reveals that thermal inactivation of cellulase (GS-CX) from salivary glands of Macrotermes subhyalinus little soldier could be described by a first-order kinetic model. The Δ-, Z-, k-values, indicate that GS-CX is heat stable and then could be utilized in pasteurization conditions, maintaining part of their biological activity. The high values obtained for activation energy (Ea) and change in enthalpy (ΔH#) indicated that a high amount of energy was needed to initiate denaturation of this carboxymethylcellulase, most likely due to its stable molecular conformation. Therefore, cellulase involves a structurally robust but temperature-sensitive enzymatic system, whose thermal denaturation is mainly under entropic control.

### References

1. Abdul S., J., T. 2015. Investigation in kinetic-thermodynamic parameters of free cellulase produced by local fungi trichoderma viride. World Journal of Pharmacy and Pharmaceutica Sciences, 4(2), 1–6
2. Awuah, G. B., Ramaswamy, H. S. Economides, A. 2007. Thermal processing and quality: Principles and overview. Ax Pharma., 2007.
3. Barrett, N. E., Grandison, A. S., & Lewis, M. J. (1999). Contribution of the lactoperoxidase system to the keeping quality of pasteurized milk. Journal of Dairy Research, 66(1), 73-80. DOI: 10.1017/S002202990003252
4. Björk L (1992). Indigenous enzyme sinnilk. Lactoperoxidase.In:Fox,F,editor. Advanced dairychemistry. 1.Proteins. London: Elsevier. p. 323-38.
5. Breznak, J. A., & Brune, A. (1994). Role of microorganisms in the digestion of lignocellulose by termites. Anna. Rev. Entomol., 39, 453-487. DOI:10.1146/annurev.en.39.010194.002321
6. Bromberg, A.; Marx, S.; Frishman, G. 2008. Kinetic study of the thermal inactivation of cholinesterase enzymes immobilized in solid matrices. Biochim, Biophys, 1784, 961-966. DOI:10.1016/j.bbabio.2008.02.018
7. Brune, A., & Stingl, U. (2005). Prokaryotic symbionts of termite gut flagellates: phylogenetic and metabolic implications of a tripartite symbiosis. In Molecular basis of symbiosis (pp. 39-60). Springer Berlin Heidelberg.
8. Chakraborty, N., Sarkar, G. M., & Lahiri, S. C. (2000). Cellulose degrading capabilities of cellulolytic bacteria isolated from the intestinal fluids of the silver cricket. Environmentalist, 20(1), 9-11.
9. Chatintrasri B, Noomhorm A (2006). Thermal inactivation of polyphenoloxidase in pineapple puree. Lebensmittel-Wissenschaft und-Technologie, 39: 492 – 495. DOI: 10.1016/j.lwt.2005.04.006
10. Clarke A.J. (1997). Biodegradation of Cellulose: Enzymology and Biotechnology. Technomic Pub. Co., Lancaster, PA, pp. 3–68.
11. D’Amico, S., Marx, J.-C., Gerday, C., & Feller, G. (2003). Activity-stability relationships in extremophilic enzymes. Journal of Biological Chemistry, 278(10), 7891-7896. DOI:10.1074/jbc.M212508200
12. Dogan M, Alkan M, Oganer Y (2000). Adsorption of methlene blue from aqueous solution onto perlite. Water, Air and Soil Pollution, 120: 229-248.
13. Dogan M, Arslan O, Dogan S (2002). Substrate specificity, heat inactivation and inhibition of polyphenol oxidase from different aubergine cultivars. Int. J. Food Sci. Technol., 37: 415-423. DOI:10.1111/j.1365-2612.2002.00580.x
14. Dogan N and Tari C. 2008. “Characterization of three-phase par-tioned exo-polygalacturonase from Aspergillus sojae with unique properties,” Biochemical Engineering Journal, 39(1): 43–50. DOI:10.1016/j.bej.2007.08.008
15. Elba P.S., Maria A.F. (2007). Bioethanol production via enzymatic hydrolysis of cellulosic biomass. Published in “The role of agricultural biotechnologies for production of bioenergy in developing countries an FAO seminar held in Rome. Available: http:// www.fao.org/biotech/seminaroc 2007.htm.
16. Epsch-Barraso, A., Van Loey, A., Hendrickx, M., &
