Partial Purification and Characterization of Cellulases by *Bacillus alcalophilus* S39 and *B. amyloliquefaciens* C23

Naser, Sohair A., M. S. Sharaf, Khadiga A. Abou –Taleb, W. A. Mashhoor  

Department of Agriculture Microbiology, Fac. of Agric., Ain Shams Univ., P.O.Box 68, Hadayek Shoubra 11241, Cairo, Egypt.

**ABSTRACT**

Maximum cellulase partial purification was achieved with 80 % (NH₄)₂SO₄ precipitation. The *Kₘ* and *Vₘₐₓ* values were calculated for CMCase, FPase & β-glucosidase. It was found that *Kₘ* values (0.92±0.28, 3.70, 0.55± 0.17 %) & (0.59±0.24, 3.86±0.39 & 0.65±0.14 %) and *Vₘₐₓ* values (4.27± 0.73, 2.50 & 6.34±1.22 U/ml) & (3.53±0.60, 2.22 & 7.42±1.04 U/ml) for *Bacillus alcalophilus* S39 and *B. amyloliquefaciens* C23, respectively. It was found that the enzymes activity has a broad pH range between 4.8 and 5. The optimum temperature of the enzymes was observed to be around 50 °C, and 97 - 98 % of the original activity was retained after heat treatment at 80 °C for 15 min. The enzymes activity was stimulated by Ca²⁺ & Co²⁺, weak inhibition of cellulases with EDTA, Cu²⁺, Acetone & Methanol, SDS & ethanol was the strongest inhibitor and the enzymes had no effect with Na⁺, Mn⁺⁺, Tween 80 and Mg²⁺. Analyses of the enzyme preparation by SDS-PAGE revealed two protein bands showing cellulolytic activity. The molecular weight of these bands was estimated to be around 66.49 and 28.47 KDa.

**Key words:** Cellulases, partial purification, enzyme characterization, enzyme secretion, *Bacillus* spp.

**Introduction**

Cellulose is commonly degraded by an enzyme called cellulase. Cellulases are relatively costly enzymes, and a significant reduction in cost will be important for their commercial use in biorefineries. Cellulase-based strategies that will make the biorefinery processing more economical include: increasing commercial enzyme volumetric productivity, producing enzymes using cheaper substrates, producing enzyme preparations with greater stability for specific processes, and producing cellulases with higher specific activity on solid substrates (Zhang et al. 2006). Cellulase is an inducible enzyme complex involving synergistic action of endoglucanase or carboxymethylcellulase (CMCase), exoglucanase and Cellobiase or β-glucosidases (Bhat 2000 and zaldivar et al. 2001). Cellulose biodegradation by cellulases and cellulosomes, produced by numerous microorganisms, represents a major carbon flow from fixed carbon sinks to atmospheric CO₂ (Falkowski et al. 2000, Melillo et al. 2002 and Berner 2003), is very important in several agricultural and waste treatment processes (Angenent et al. 2004, Das & Singh 2004, Haight 2005 and Schloss et al. 2005) and could be widely used to produce sustainable biobased products and bioenergy from less costly renewable lignocellulosic material to replace depleting fossil fuels and reduce environmental pollution (Demain et al. 2005, Moreira 2005 and Reddy & Yang. 2005). The production of cellulases by wild type cells of *Bacillus pumilus* (Kotchoni and Shonukan 2002), *Cellulomonas biazotea* (Rajoka 1998) and *Trichoderma aureoviride* (Zaldivar et al. 2001) in liquid media did not exceed 1.5 U/ml. Production of filter paper cellulase (FPase), endo-β-glucanase and β-glucosidase by *Cellulomonas biazotea* was investigated during growth on different cellulosic substrates (Rajoka and Malik 1997). Bacteria such as *Clostridium thermocellum*, *Ruminococcus albus*, *Streptomyces* sp. and actinomycetes (*Thermoactinomyces* sp., *Thermomonospora curvata* and *Streptomyces* sp.) have also been reported to produce cellulose (Lamed & Bayer 1988 and Ohara 2000). However, pure cellulases from bacteria have not yet been commercially produced and standardized enzyme preparations are not available. Moreover, the production of cellulase by most of these organisms is under the control of catabolite repression.

The present study was carried out to screen extracellular cellulolytic enzymes from two bacterial strains (*Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* C23) and characterization of cellulases activities.

**Materials and Methods**

*Isolation and identification of cellulase producing bacteria*

The bacterial isolates used in this study were collected from compost and soil. Isolates were purified and screened for their cellulase activity using carboxymethyl cellulose (CMC) agar medium. The CMC agar plates

**Corresponding Author:** M. S. Sharaf, Department of Agriculture Microbiology, Fac. of Agric., Ain Shams Univ., P.O.Box 68, Hadayek Shoubra 11241, Cairo, Egypt.  
E-mail: msaidsharaf@yahoo.com
of isolates obtained from soil and compost were incubated at 30 and 45 °C respectively for 72 h to allow for the secretion of cellulases. After incubation, the agar plates were flooded with an aqueous solution of Congo red (1%) for 15 min., the Congo red solution was then poured off and the plates were washed with 1 M NaCl for 15 min., positive cellulytic isolates were detected based on the clear zone of hydrolysis on CMC agar plates. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest cellulase activity producer. The largest ratio was assumed to contain the highest activity. The two most efficient bacterial isolates of the selected cellulytic producers were distinguished as potent cellulase were picked up for further identification according to their cultural, morphological and biochemical characteristics based on Bergey's Manual of Systematic Bacteriology (Claus & Berkeley 1986) and Biolog Automated System was used.

Media used

Nutrient agar (Difco Manual 1984) was used for the maintenance of bacterial strains. Carboxymethyl cellulose medium recommended by Ray et al. (2007) was used for cellulases production. It has the following composition (g/l): Carboxymethylcellulose (CMC), 10; Tryptone, 2; KH2PO4, 4; Na2HPO4, 4; MgSO4.7H2O, 0.2; CaCl2.2H2O, 0.001; FeSO4.7H2O, 0.004; Agar, 15 and pH adjusted to 7.

Inoculum preparation and fermentation process

For preparation of standard inoculum, both strains were cultured in nutrient broth individually at 30 °C for 24 h where an average viable count of 3.5 - 4.3 ×10^6 viable cells / ml culture broth was obtained. This was used as the inoculum for the production medium. Fermentation was carried out in 250 ml plugged Erlenmeyers flasks, each containing 50 ml sterile production medium and inoculated with 3% of standard inoculum (containing about 3.5 ×10^8 and 4.3 ×10^6 cells / ml for B. amyloliquefaciens C23 and Bacillus alcalophilus S39, respectively). The inoculated flasks were incubated at 30 and 45 °C for strain S39 and strain C23, respectively on rotary shaker at 150 rpm for 72 h.

Preparation of crude enzyme

After incubation, cultures were centrifuged at 1600 g for 15 min at 4°C and supernatants were used as source of crude enzymes. The crude enzyme solution was utilized for determination of enzyme activities (Kotechoni et al. 2005).

Partial purification of cellulase

Ammonium sulphate precipitation

The proteins in the crude preparation were precipitated by the addition of solid ammonium sulphate to (20 - 80%) saturation. The precipitate was allowed to form at 4° C for 24 h, and was collected by centrifugation at 4000 g in a cold centrifuge at 4° C for 30 min.

Dialysis

The precipitate collected from each source was dissolved individually in small amount of sodium acetate buffer (0.2 M) at pH 5.5 and were dialyzed against the same buffer overnight at 4°C with two times changes of the buffer.

Enzyme assays procedures

a) Carboxymethyl-cellulase(CMCase) activity

CMCase activity was assayed using a method described by Mandels and Weber (1969). The activity was estimated using 1 % solution of carboxymethyl cellulose (CMC) in 0.05 M citrate buffer (pH 4.8) as substrate. The reaction mixture contained 1 ml citrate buffer, 0.5 ml of substrate solution and 0.5 ml of suitably diluted enzyme solution. The reaction was carried out at 50°C for 30 min. The amount of reducing sugar released in the hydrolysis was measured. One unit of CMCase activity was expressed as 1 μ mol of glucose liberated per ml enzyme per minute.
b) Filter-paperase (FPase) activity

The activity of FPase was assayed according to the method explained by Mandels and Weber (1969). This method is similar to the CMCase assay method, but the substrate was Whatman No. 1 filter paper strip (1 x 6 cm) soaked in 1 ml 0.05 M sodium citrate buffer (pH 4.8). The samples were incubated with 0.5 ml enzyme solution at 50°C for 1 h, the reducing sugars liberated during growth were determined. One unit of FPase activity was determined as 1 μ mol of glucose liberated per ml enzyme per minute.

c) β-Glucosidase activity

One-tenth ml of the culture supernatant was incubated with 0.5 ml of 0.05 M acetate buffer (pH 5) containing 2.5 mg cellobiose. After incubation at 50 ºC for 10 min the glucose released was measured by the glucose oxidase peroxidase method (Zaldívar et al. 2001).

Determination of reducing sugars

The total amount of reducing sugars was determined using potassium ferricyanide method, as described by Park and Johnson (1949).

Determination of total protein

The protein concentration of the crude as well as that of the partial purified enzyme was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Characterization of the Enzymes

Substrate concentration

The effect of substrate on cellulases activity was studied for the following parameters:

a) Determination of $V_{max}$ and $K_m$

For assaying CMCase, CMC was used as substrate with variable amounts (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5 & 20 mg/ml) of 1% CMC, filter paper was used as substrate for assaying FPase with different concentrations (0, 12.5, 25, 37.5, 50, 62.5, 75, 87.5 & 100 mg/ml) and cellobiose was used as substrate for estimating β-Glucosidase with varying concentrations (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 & 10 mg/ml). The data obtained were plotted according to Lineweaver-Burk, Eadie-Hofstee and Hanes-Wolf plot as described by Price and Dwek (1986).

b) Determination of specificity constant

The specificity constant of the cellulase was calculated as follows: $V_{max}/K_m$.

Optimal pH for cellulases activity

The enzyme was mixed with substrates at different pH levels (pH 3 to 8) for 30, 60 and 10 min for CMCase, FPase and β-Glucosidase, respectively to assay its optimal pH.

Optimal temperature for cellulases activity

The enzyme was incubated with substrates at various temperatures ranged from 20 to 80°C for 30, 60 and 10 min for CMCase, FPase and β-Glucosidase, respectively to estimate the optimal temperature enzyme activity.

Effect of heat stability on cellulases activity

The enzyme was incubated in the standard buffer at 40, 50, 60, 70 and 80°C for 15 min and reaction tubes were placed in ice. Thereafter the activity was assayed.
Effect of chemical reagents on enzyme activity

The effect of chemical reagents on reaction velocity catalyzed by cellulase enzymes complex were determined using some chemical compounds such as: EDTA, SDS, Tween 80 (0.1%, final concentration), metal ions such as: Ca++, Co++, Cu++, Mg++, Mn++ and Na+ (1 mM, final concentration) and some organic solvents (20 %, v/v; final concentration) such as: acetone, ethanol and methanol on the cellulase activity was studied using 1 % CMC as substrate (Korish, 2003). The substrate-chemical reagents mixture was incubated at room temperature for 1 h before it was used in enzyme assay.

Molecular Weight of protein enzyme

Polyacrylamide gel analysis was used to determine the molecular weight of the cellulase enzyme. The gel was analyzed by gel documentation Alpha Ease FC 4.0 software (Alpha Innotech Corp. San Leandro, CA). Then the molecular weight of individual enzyme fractions was determined by referring the molecular weight of the marker.

Results and Discussion

Screening and Identification of Cellulytic Producers

Screen of bacterial isolates for cellulases activities was conducted by using the Congo red test as a preliminary study for selecting cellulases producers. After 72 hrs of incubation, all 48 isolates collected from soil and 47 isolates obtained from compost showed signs of growth on CMC agar and demonstrated positive results in the Congo red test. Since the sole carbon source in CMC agar was carboxymethyl cellulose, therefore the result of the test was a strong evident that cellulases was produced in order to degrade cellulose. Regarding to their cultural and morphological characteristics based on Bergey's Manual of Determinative Bacteriology (1984), isolates were belonged to the genus Bacillus. They were rod-shaped, aerobic, spore forming, gram-positive, acid fastness, motile and according to Claus & Berkeley (1986). The two bacterial strains were identified by Biolog Automated System as Bacillus alcalophilus S39 and Bacillus amyloliquefaciens C23 isolated from soil and compost respectively, which gave the highest ratios of clear zone diameter to colony diameter being 3.52 and 3.50 cm, respectively for cellulose degradation. Bacillus spp. are common in compost and produce the alkaliphilic properties of cellulases (Dees & Ghiorse, 2001 and McCaig et al. 2001). In addition, that isolated thermophilic Bacillus strains with cellulytic activities, similarly cellulytic properties of bacterial species like Pseudomonas, Cellulomonas, Bacillus, Micrococcus, Cellovibrio and Sporophytophaga spp. (Mawadza et al. 2000 and Immanuel et al. 2006).

Partial purification of cellulase enzyme through ammonium sulfate (NH₄)₂SO₄

Cellulases produced by Bacillus alcalophilus S39 and B. amyloliquefaciens C23 were partially purified by the addition of ammonium sulphate (NH₄)₂SO₄ into the crude enzyme extract followed by dialysis. Maximum cellulases partial purification was achieved with 80% (NH₄)₂SO₄ precipitation. Data are recorded the specific activity of CMCase being 1.20 and 1.12, with 2.13 and 2.00 fold increase after purification, FPase being 0.73 and 0.64, with 2.61 and 2.34 fold increase after purification and β-glucosidase was 1.68 and 1.62, with 2.00 and 1.97 fold increase after purification for Bacillus alcalophilus S39 and B. amyloliquefaciens C23, respectively (data not shown). Obtained results are in disagreement with those obtained by Shafique et al. (2004) found that the exoglucanase produced by Bacillus subtilis was partially purified by the addition of (NH₄)₂SO₄ into the crude enzyme solution. Maximum exoglucanase purification was achieved with 20% (NH₄)₂SO₄ precipitation. The analysis of residual enzyme protein yielded 0.094 and 0.12 mg/ml protein in supernatants and residues, respectively. Specific activity of exoglucanase was 230.93 and 229.95 for residue and the supernatant, with 1.51 and 1.50 fold increases after purification, respectively. Partial purification with ammonium sulphate showed that the protein fraction obtained at 20 - 40% ammonium sulphate saturation was most suitable for enzymes recovery (Fadel, 2000).

Characterization of the cellulase enzyme

Substrate concentration

To study the effect of substrate concentration on the velocity of the partially purified enzyme the following experiment was run. The $K_m$ and $V_{max}$ values were determined by using the different equations. This was carried out to evaluate the relation between nonlinear regression using Michaelis-Mentent Plot equation and
its derivatives: Hanse-Woolf Plot, Eadie-Hofstee Plot and Lineweaver-Burk Plot. Results are presented in Table 1. The results for the different enzymes are disused individually: a) CMCase exhibited an affinity toward carboxymethyl cellulose (CMC) substrate where, $K_m$ were 0.92±0.28 & 0.59±0.24 %; and $V_{max}$ were 4.27±0.73 & 3.53±0.60 U/ml for Bacillus alcalophilus S39 and B. amyloliquefaciens C2, respectively. b) FPase exhibited an affinity toward filter paper as substrate since, $K_m$ values were 3.70 & 3.86±0.39 %; and $V_{max}$ were 2.50 & 2.22 U/ml for Bacillus alcalophilus S39 and B. amyloliquefaciens C2, respectively. c) $\beta$-glucosidase exhibited an affinity toward cellulobiose as substrate since, the values of $K_m$ was 0.55± 0.17 & 0.65±0.14 %; and $V_{max}$ were 6.34±1.22 & 7.42±1.04 U/ml for Bacillus alcalophilus S39 & Bacillus amyloliquefaciens C2, respectively. For most enzyme catalyzed reactions, the $K_m$ and $V_{max}$ are important parameters and these depend on several rate constants. Similar to our observations, Waksman (1991) found that $K_m$ value of 8.7 mg/ml was obtained for the endoglucanase from Sclerotinia sclerotiorum. Lin and Stutzenberger (1995) also produced $\beta$-1,4 endoglucanase of Thermophilic actinomyceete and found that $K_m$ value equal to 7.33 mg/ml.

### Table 1: Different techniques for calculating $K_m$ and $V_{max}$ values for partially purified Bacillus alcalophilus S39 and B. amyloliquefaciens C2, cellulases.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Cellulase</th>
<th>Lineweaver-Burk Plot</th>
<th>Eadie-Hofstee plot</th>
<th>Hanse-Woolf Plot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (U/ml)</td>
<td>$V_{max}$ (%)</td>
<td>specificity constant</td>
<td>$K_m$ (U/ml)</td>
</tr>
<tr>
<td>CMCase</td>
<td>2.4686 x + 0.1999</td>
<td>-8.9358x + 4.2245</td>
<td>y = 0.234x + 2.1594</td>
<td></td>
</tr>
<tr>
<td>FPase</td>
<td>14.8 x + 0.3996</td>
<td>-36.009x + 2.481</td>
<td>y = 0.3985x + 14.755</td>
<td></td>
</tr>
<tr>
<td>$\beta$-glucosidase</td>
<td>0.3685x + 0.1585</td>
<td>-3.58x + 6.4435</td>
<td>y = 0.1335x + 0.951</td>
<td></td>
</tr>
<tr>
<td>Bacillus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alcalophilus</td>
<td></td>
<td></td>
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<tr>
<td>S39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMCase</td>
<td>2.009 x + 0.2423</td>
<td>-8.6355 x + 3.5275</td>
<td>y = 0.3043x + 1.483</td>
<td></td>
</tr>
<tr>
<td>FPase</td>
<td>4.13 x + 4.98</td>
<td>0.59 3.53 5.98</td>
<td>y = 0.4549 x + 17.252</td>
<td></td>
</tr>
<tr>
<td>$\beta$-glucosidase</td>
<td>8.265 x + 0.4299</td>
<td>-38.628 x + 2.2226</td>
<td>y = 0.6459 x + 17.252</td>
<td></td>
</tr>
<tr>
<td>Bacillus</td>
<td></td>
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<tr>
<td>amyloliquefaciens</td>
<td></td>
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<tr>
<td>C2</td>
<td></td>
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<td></td>
</tr>
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<td>0.3043 x + 1.483</td>
<td>-8.6355 x + 3.5275</td>
<td>y = 0.3043x + 1.483</td>
<td></td>
</tr>
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<td></td>
</tr>
</tbody>
</table>

### Effect of pH on cellulase activity

It was observed that the enzymatic activity has a broad pH range from 3 to 9. The used buffer for CMCase & FPase was 0.05 M citrate buffer (pH 4.8) while 0.05 M acetate buffer (pH 5) for $\beta$-glucosidase. Results are illustrated in Fig.1 show that the optimum pH from 4.8 to 5 achieved the highest activities for CMCase and FPase with 100% relative activity, while the highest $\beta$-glucosidase activity was found at pH 5 with 100% relative activity. These data indicated that the enzyme was not an alkaline enzyme because it loosing the activity at pH from 7 to 9. Results are in line with those of André et al. (2005) and Onsori et al. (2005) found that endoglucanase from Bacillus pumilus was shown to be active at a pH ranging from pH 5 – 8. A previous report Kotchoni et al. (2006) also found that the enzyme is not an alkaline one because of lost activity more than 65% of its activity at pH 6 to 7.

**Fig.1.** Effect of pH range on cellulases activity produced by B. alcalophilus S39 and B. amyloliquefaciens C2,
Effect of temperature on cellulases activity

The enzyme reaction velocity was increased by increasing temperature from 20 to 50°C, and then decreased at 60°C. The optimum temperature for Bacillus alcalophilus S39 and B. amyloliquefaciens C2s was 50°C, which dropped at 70 and 80°C. At 50°C all the three enzymes (CMCase, FPase and β-glucosidases) show maximum activities, but FPase was active between 40 and 50°C (Fig. 2). These data are in agreement with findings obtained by Kotchoni et al. (2003) who found that the cellulases from the wild type Bacillus pumilus and BpCR1 6 mutant displayed its optimum activity at 50°C, which dropped to 80 % at 70°C and further to 30 % at 80°C both on CMC and cellobiose.

![Fig. 2. Effect of temperature on cellulases activity produced by B. alcalophilus S39 and B. amyloliquefaciens C2s.](image)

Effect of heat stability on cellulases activity

The thermostability of the enzyme was studied by heating the enzyme at different temperatures (30-80°C) for 15 min. Results are illustrated by Fig. 3 clearly show that cellulase enzymes were stable at temperature 30°C for 15 min. The decreasing in the thermal stability of cellulase enzymes was first recorded after holding the enzyme at 40°C for 15 min. On the other hand, the enzymes was destroyed by heating at 80°C for 15 min, as a result, (2.55, 5.49 & 1.32%) and (9.29, 18.37 & 3.32 %) of the original activity of CMCase, FPase and β-glucosidases by Bacillus alcalophilus S39 and B. amyloliquefaciens C2s, respectively were retained after heat treatment at 80°C for 15 min. From previous data it can be concluded that 30°C is the suitable degree for enzyme storage stability. This may be due to that, no autodigestion of cellulase enzymes occurred during incubation period of 15 min at 30°C. At all higher temperature, the decrease in enzymes activity may be due to autolysis of cellulase enzymes during incubation. Similarly, Kotchoni et al. (2003) reported that 40 % of the initial activity was retained after heat treatment at 80°C while the wild type for Bacillus pumilus enzyme lost its activity under similar conditions.

Effect of chemical reagents on cellulases activity

An experiment was designed to study the influence of various metal ions and some substances on cellulases activity which is presented as the percentage of CMCase, FPase and β-glucosidases activity compared to the control condition. Three chemical compounds namely, (EDTA [ethylenediaminetetraacetic acid], SDS [Sodium dodecyl sulphate] & Tween 80), six metal ions namely, (Ca++, Co++, Cu++, Mg++, Mn++ & Na+) and three organic solvents namely, Acetone, Ethanol and Methanol, were studied. Results were illustrated by Fig. 4 show that the ions of (Cu++, Co++ & Mg++), (Ca++, Co++ & Tween 80) for Bacillus alcalophilus S39 & B. amyloliquefaciens C2s, respectively enhanced the enzyme activity. However, ions of (Na+, Mn++ & Tween 80) and (Na+, Mn++ & Mg++) for Bacillus alcalophilus S39 & B. amyloliquefaciens C2s, respectively have no effect on the enzymes activity. While, EDTA, the ions of copper (Cu+), acetone and methanol were inhibited the enzyme activity, and strong inhibition of cellulase was observed with SDS & ethanol. The strong inhibition was observed with SDS at 10 mM on endoglucanase from Bacillus circulans, also found that organic solvents (methanol, ethanol, acetonitrile, ethylacetate and dimethylsulfoxide) caused inhibition of the activity of cellulase from Chalara paradoxa at different degrees Lucas et al. (2001). Cu+++ and Mg++ were found to increase the activity of the enzyme Kotchoni et al. (2006). These cations might be probably involved in the protection of the enzyme or strengthening of the active site thereby maintaining the confirmation of the enzyme in active state, whilst Cu++, Cd++ and Ni++ were found to inhibit the activity. Cu++, Cd++ and Ni++ have been reported as heavy metals and generally toxic to some extent to organisms (Sunkar et al. 2003 and Rani et al. 2004). Ajayi et al.
2007 show that Mg$^{++}$, Ca$^{++}$, Na$^{+}$ and K$^{+}$ were stimulatory to activity of the enzyme while small quantities of HgCl$_2$, EDTA and DNP were inhibitory suggests that the enzyme may be cation requiring. Stimulation of cellulases by cations as well as their inhibition by EDTA, DNP and HgCl$_2$ has been reported by (Akiba et al. 1995 and Ajayi et al. 2003).

![Fig.3. Effect of heat-stability on cellulases activity produced by B. alcalophilus S39 and B. amyloliquefaciens C2$_3$.](image)

![Fig.4. Effect of chemical reagents on relative cellulases activity produced by B. alcalophilus S39 and B. amyloliquefaciens C2$_3$.](image)

**Protein banding patterns**

**SDS PAGE analysis**

Protein analysis by SDS-PAGE of 2 strains (Bacillus alcalophilus S39 and B. amyloliquefaciens C2$_3$) illustrated in Fig. 5 and Table 2. It was found that various in total number and density protein fractions (polypeptides) among to 2 strains. Bacillus alcalophilus S39 and B. amyloliquefaciens C2$_3$ revealed 17 polypeptides, 7 of protein marker (4 for Bacillus alcalophilus S39 & 3 for B. amyloliquefaciens C2$_3$) as unique and 5 monomorphic (66.49, 60.82, 28.47, 22.88 & 12.31 KDa). On the other hand the cellulase purified appeared 3 polypeptides with molecular weight 66.49, 28.47 and 14.23 KDa. Whereas both strains revealed to 2 polypeptides from 3 (66.49 & 28.47 KDa). In addition 2 species were differed in density (protein content) of them. The previous data indicated that Bacillus alcalophilus S39 and B. amyloliquefaciens C2$_3$ were identified by two protein marker with molecular weight 66.49 and 28.47 KDa. Fig. 6 illustrated cryptogram of protein patterns for B. alcalophilus S39 and B. amyloliquefaciens C2$_3$ compared with cellulase purified. It was observed
that various in peak of polypeptides for each of them and area (density) as well as protein content of each polypeptides (intensity). On the other hand two Bacillus strains (Bacillus alcalophilus S39 and B. amyloliquefaciens C23) included 2 polypeptides from 3 polypeptides of purified enzyme. The cellulase purified enzyme content 3 isozymes, while B. alcalophilus S39, B. amyloliquefaciens C23, content (2 and 2 isozymes) respectively, only in addition they differ in intensity.

Fig. 5. SDS-PAGE gel electrophoresis of proteins patterns.

Samples: 100 µg protein per well (M- Marker 1- B. alcalophilus S39 2- B. amyloliquefaciens C23, 3- Cellulase purified).

Table 2: Protein content and fractions of Bacillus strains compared with cellulase purified by SDS-PAGE.

<table>
<thead>
<tr>
<th></th>
<th>Cellulase purified</th>
<th>Bacillus alcalophilus S39</th>
<th>Bacillus amyloliquefaciens C23</th>
<th>Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of polypeptides (TAF)</td>
<td>3</td>
<td>9</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Molecular weight</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Unique</td>
</tr>
<tr>
<td>Protein content in fraction %</td>
<td>75.53</td>
<td>66.49</td>
<td>60.82</td>
<td>Monomorphic</td>
</tr>
<tr>
<td>Density</td>
<td>1.5</td>
<td>21.5</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Molecular weight</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Unique</td>
</tr>
<tr>
<td>Protein content in fraction %</td>
<td>47.50</td>
<td>54.37</td>
<td>43.12</td>
<td>Monomorphic</td>
</tr>
<tr>
<td>Density</td>
<td>0.4</td>
<td>2.50</td>
<td>2.1</td>
<td></td>
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<tr>
<td>Molecular weight</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Unique</td>
</tr>
<tr>
<td>Protein content in fraction %</td>
<td>41.29</td>
<td>38.94</td>
<td>8.3</td>
<td>Monomorphic</td>
</tr>
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Clusters analysis

Fig. 7 illustrated Protein enzyme among microorganisms and purified enzyme were estimated by similarity coefficient matrix based on SDS-PAGE bands scored. Pair wise values of this similarity coefficient ranged from 96 to 100 for Bacillus strains and cellulase purified. The dendrogram was constructed based on the dissimilarity matrix, using UPGMA method. Fig. 7 illustrated that two groups contained, the first major group contained only B. alcalophilus S39 with similarity 100 % with second group. The second major cluster contained B. amyloliquefaciens C23 and cellulase purified with 96.8 %.
Fig. 6. Gel documentation software analysis showing cryptogram cellulase protein patterns, distance and intensity of polypeptides for B. *alcalophilus* S39 & B. *amyloliquefaciens* C23.

Fig. 7. Dendrogram showing cellulase protein enzyme relationships among B. *alcalophilus* S39 and B. *amyloliquefaciens* C23 showing L1= Cellulase purified, L2= B. *alcalophilus* S39, L3= Bacillus *amyloliquefaciens* C23.

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**References**


