Purification and characterization of highly thermostable amylopullulanase from a thermophilic, anaerobic bacterium Clostridium thermosulfurogenes SVM17

Mrudula, Soma*, Gopal Reddy2 and Seenayya, Gunda3

1 Department of Microbiology, M. G. R. College, Dr. M. G. R. Nagar, Hosur, T.N, 635 109, India.
2 Department of Microbiology, Osmania University, Hyderabad, 500 007, A.P, India.
E-mail: somamrudula@hotmail.com

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ABSTRACT

A highly thermostable amylopullulanase was purified to homogeneity from the culture filtrate of the Clostridium thermosulfurogenes SVM17. On SDS-PAGE, the purified fraction having both amylase and pullulanase activities were observed as a single band. The molecular weight of the purified amylopullulanase on SDS-PAGE was 97 kDa. The optimum temperature for both amylase and pullulanase was 70 °C. The enzyme was completely stable at 70 °C for 2 h. The presence of 5% starch increased the thermal stability of the enzyme at 100 °C up to 2 h. Both amylase and pullulanase activities were optimum at pH 5.5 to 6.0 and were stable over a pH range of 4.0 to 6.5. The TLC analysis of the reaction products on starch showed that maltose was the main product along with trace amounts of glucose. The analysis of hydrolysis product of pullulan showed that maltotriose was the main product. At 5 mM concentration, Mn2+ and Ag+ strongly stimulated both amylase and pullulanase activities, whereas Mg2+, Ca2+, Cu2+, Fe2+, Zn2+, Hg22+, EDTA, Cd2+ and Li+2 inhibited both amylase and pullulanase activities. When the concentration of metal ions was increased from 5 to 10 mM, a further increase in amylase activity was observed in the presence of Ni2+, Mn2+ and Co2+. Where as substantial decrease was observed at 10 mM concentration of Ag+, Pb2+ and Ca2+.

Keywords: Amylase, pullulanase, Clostridium thermosulfurogenes SVM17, purification, characterization

INTRODUCTION

Malto and maltooligosaccharides find wide range of applications in food, beverage, pharmaceutical and fine chemical industries (Fogarty and Kelly, 1994; Sivaramakrishnan et al., 2006). They are produced by hydrolysis of starch using amylases. The majority of amylases so far reported are optimally active at moderate temperatures (Haki and Rakshit, 2003; Zareian et al., 2010; Shafiei et al., 2010) and thermally unstable (Shen et al., 1988). Therefore a high value is placed on extreme thermostability and thermostability of these enzymes in the bioprocessing of starch (involves liquefaction and saccharification). Thermostable amylases are of special interest as they could be used for saccharification processes occurring at high temperatures (Gomes et al., 2003; Soni et al., 2005; Saxena et al., 2007). The advantages of using thermostable amylases in industrial processes include the decreased risk of contamination, cost of external cooling and increased diffusion rate (Lin et al., 1998). It would be advantageous to have microorganisms that produce thermostable enzymes having properties of both amylase and pullulanase, because it cleaves both α-1,4-linkages in starch and amylase and α-1,6-linkages in pullulan and branched polysaccharides, respectively. Such type of endo acting enzymes has been designated as amylopullulanases (Mrudula, 2010; Zareian et al., 2010; Mrudula et al., 2011).

In this respect, efforts have been made to isolate microorganisms (Swamy and Seenayya, 1996a) that produce amylopullulanase which is considered to be most important source for industrial applications. In the present study, we report on purification and characterization of amylopullulanase produced by Clostridium thermosulfurogenes SVM17.

MATERIALS AND METHODS

Microorganism and culture conditions

The bacterial strain used in the present study was isolated in our laboratory and identified as Clostridium thermosulfurogenes SVM17 (Swamy and Seenayya, 1996a; Mrudula et al., 2010; Mrudula et al., 2011). The organism was cultivated anaerobically at 60 °C in 120 mL serum vials that contained 20 mL of pre-reduced peptone yeast extract (PYE) medium (Swamy and Seenayya, 1996b).

The stationary growth phase cultures were harvested by centrifugation at 10,000 x g for 15 min at 4 °C. The supernatant was used as the extracellular enzyme preparation for further purification processes. The bacterial pellet was washed twice with double distilled water.
water, suspended in distilled water and used for the estimations of cell bound enzyme activity.

**Purification of enzymes**

Analytical grade solid ammonium sulphate (at 70 % saturation) was added to 1 L of the supernatant (4 °C). After incubating overnight at 4 °C, the precipitate formed was separated by centrifugation (15,000 \times g, 20 min at 4 °C). The collected precipitate was dissolved in 25 mL of acetate buffer (0.1 M, pH 5.5) and dialysed against the same buffer. The undissolved precipitate in the dialysed sample was removed by centrifugation (15,000 \times g, 20 min at 4 °C). The collected supernatant was assayed for amylolytic activity and protein content. The enzyme present in the supernatant solution was concentrated by freeze drying in a freeze drier (Heto).

The concentrated enzyme solution was applied on to sephadex G-200 (Sigma) column (2 \times 100 cm), previously equilibrated with sodium acetate buffer (0.1 M, pH 5.5), and eluted with the same buffer. The concentrations of protein in the fractions were measured by the absorbance at 280 nm and enzyme assay was carried out. The active fractions were pooled, concentrated and subjected to SDS-PAGE to determine the number of bands.

**Enzyme assays**

Amylase and pullulanase activities were measured by incubating 0.5 mL of appropriately diluted enzyme source with 0.5 mL of 1 % (w/v) starch (for amylase assay) and pullulan (for pullulanase assay), respectively in 2 mL of 0.1 M acetate buffer (pH 5.5) at 70 °C for 30 min. After incubation, reaction was stopped and reducing sugars released by enzymatic hydrolysis of soluble starch and pullulan were determined by addition of 1 % (w/v) starch and pullulan were assayed as described.

The end products formed as a result of starch and pullulan hydrolysis were analyzed by thin layer chromatography. The reaction mixture containing 0.5 mL of enzyme purified solution, 0.5 mL of 2 % soluble starch and pullulan, respectively in 2 mL of 0.1 M sodium acetate buffer (pH 5.5) were incubated at 70 °C for 30 min and the reaction was stopped by heating the reaction mixture in boiling water bath for 5 min. 20 µL of reaction mixtures were spotted separately along with glucose, maltose, maltooltriose as reference standards on to precoated silica gel plates (Merck, Art No. 5553, Germany) previously activated at 100 °C for 30 min. The plates were developed in a saturated chromatographic chamber at room temperature with a solvent system of butanol:acetic acid:water [(3:1:1) by volume (Kim et al., 1995)]. After developing the hydrolysates, the sugar spots were visualized by spraying with a mixture containing aniline (4 mL), diphenylamine (4 g), 85% ortho-phosphoric acid (30 mL) and acetone (200 mL). The plates were dried in an oven at 105 °C for 1 h. The sugar spots of the hydrolysates were identified by comparing their Rf values with those similarly obtained for standard sugar spots.

**Effect of temperature on enzyme activity and stability**

The relative enzyme activities were measured by incubating the reaction mixture at various temperatures for 30 min. To study temperature stability, the enzyme samples were incubated at various temperatures and samples were withdrawn for assaying the remaining activities at appropriate time intervals. Effect of different concentrations of starch (0, 1, 3 and 5%) on thermal stability of the enzyme was determined by incubating the reaction mixtures at 100 °C and the remaining activities were assayed as described.

**Effect of pH on enzyme activity and stability**

The relative enzyme activities were measured by holding the enzyme reaction mixture at various pH values (2.0-10.0) for 30 min at 70 °C. The buffers (0.1 M) used in the reactions were: glycine hydrochloride buffer (pH 2.0-3.0), sodium acetate buffer (pH 4.0-6.0), sodium phosphate buffer (pH 6.0-8.0) and sodium glycine buffer (pH 9.0-10.0). To determine pH stability, the enzyme solution in 0.1 M acetate buffer (pH 5.5) was preincubated at various pH values at 70 °C for 2 h and then the residual activities were assayed as described.

**Effect of metal ions on enzyme activity**

The effect of metal ions in enzyme activities were studied by incorporating different metal ion concentration in a reaction mixture containing the enzyme and metal ions incubated at 70 °C for 30 min and then the residual activities were measured under standard assay conditions.

**RESULTS AND DISCUSSION**
Purification of the enzyme

*C. thermosulfurogenes* SVM17 showed 951 and 468 U of amylase and pullulanase activities (both extracellular and cell bound), respectively per liter of culture broth. After centrifugation the crude culture supernatant showed approximately 363 U of amylase and 263 U of pullulanase per liter. Salting out of the enzyme with ammonium sulfate, resulted in the concentration of protein and an increase in the specific activity of pullulanase from 0.15 to 0.75 U/mg and for amylase from 0.31 to 1.04 U/mg proteins with a recovery of 56.20% of pullulanase and amylase, respectively (Table 1). The elution profile of the enzyme on sephadex G 200 column is shown in Figure 1. Overall the specific activities of amylase and pullulanase were increased to 44.45 and 68.73 fold with an yield of 10.73% and 16.28%, respectively. The preparation was shown to be a homogeneous by SDS-PAGE (Figure 2).

### Table 1: Summary of purification of *Clostridium thermosulfurogenes* SVM17 amylopullulanase enzymes.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg of protein)</th>
<th>Purification factor</th>
<th>Yield (%)</th>
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<tr>
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<td>3122</td>
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<td>468</td>
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<td>349</td>
<td>363</td>
<td>263</td>
<td>1.04</td>
<td>0.75</td>
</tr>
<tr>
<td>dialysis</td>
<td></td>
<td></td>
<td></td>
<td>3.4</td>
<td>5</td>
</tr>
<tr>
<td>Gel filtration using sephadex G200</td>
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<td></td>
<td></td>
<td>38.2</td>
<td>56.2</td>
</tr>
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<td>295.5</td>
<td>183.82</td>
<td>11.32</td>
<td>7.04</td>
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<td>68.73</td>
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<td></td>
<td>10.73</td>
<td>16.28</td>
</tr>
</tbody>
</table>

**Figure 1:** Elution profile of amylase and pullulanase on sephadex G-200.

**Action pattern of the enzyme**

The substrate hydrolysis end products of *C. thermosulfurogenes* SVM17 amylase on starch showed that maltose as the main product along with trace amounts of glucose indicating the presence of amylase activity. When pullulan was hydrolysed, only maltotriose was observed as hydrolysis product. This indicates that the strain SVM17 had pullulanase activity (Figure 3).

**Figure 2:** Analysis of purified amylolytic enzyme on SDS-PAGE, gel stained with silver staining. Lane 1 indicates the complete protein profile of extra cellular amylolytic enzyme. Lane 2 indicates the purified amylolytic enzyme, Lane 3 and 4 indicates different fractions eluted by column chromatography and Lane 5 indicates the standard molecular markers.

Since the strain showed single band on SDS-PAGE and having both amylase and pullulanase activities as evidenced by TLC, the enzyme is designated as endo-acting type i.e., pullulanase type II.
(Spreinat and Antranikian, 1990) or amylopolllullanase (Saha and Zeikus, 1987).

**Molecular weight of purified amylopolllullanase**

The molecular weight of purified enzyme determined by SDS-PAGE was 97 kDa (Figure 2). The molecular weight of amylopolllullanase from different organisms ranged between 40 and 450 kDa. The molecular weights of amylopolllullanase from *Bacillus circulans* F-2 (Sata et al., 1989), *Thermus aquaticus* YT-1 (Plant et al., 1986), *Pyrococcus furiosus* (Brown and Kelly, 1993), *P. furiosus* (Dong et al., 1997), *P. woesei* (Rudiger et al., 1995), *C. thermosulfurogenes* EMI (Spreinat and Antranikian, 1990), *Bacillus* sp. KSM 1378 (Ara et al., 1996) and *Sulfotobus acidocaldarius* (Deweer et al., 2000) were 220, 83, 110, 90, 102, 210 and 95 kDa, respectively. The molecular weight (97 kDa) of amylopolllullanase by *C. thermosulfurogenes* SVM17 is within the range and closer to that of *C. thermosulfurogenes* EMI amylopolllullanase. The amylopolllullanase produced by *Thermococcus profundus* had a very low molecular weight of 43 kDa (Kwak et al., 1998), where as the enzyme from *Thermoaerobacter* strain B6A (Saha et al., 1990), has a high molecular weight of 450 kDa. Most of the amylopolllullanase have shown to be glycoproteins (Saha et al., 1990; Brown and Kelly, 1993). The enzymes having attached sugars tend to have high molecular weights depending on the type and amount of sugars.

**Figure 3:** TLC of the hydrolysed products of starch and pullulan formed by the action of purified SVM17 amylolytic enzyme. Lane 1, 2 and 3 indicates standard glucose, maltose and maltotriose, respectively. Lane 4 shows the hydrolytic products of starch, lane 5 shows the hydrolytic product of pullulan and lane 6 represents the standard sugars G1 (glucose), G2 (maltose) and G3 (maltotriose).

**Thermostability the enzyme**

The effect of temperature on amylase and pullulanase activities of the enzyme is shown in Figure 4. Both amylase and pullulanase activities displayed a broad temperature range from 40 to 100 °C with the maximum at 70 °C. Gomes et al. (2003) reported the maximum amylase and pullulanase activities at 85 and 80 °C, respectively.

**Figure 4:** Effect of temperature on enzyme activity.

Both enzyme activities were assayed with 1 % respective substrate in 0.1 M acetate buffer (pH 5.5). Incubations were carried out at indicated temperatures for 30 min. The thermal stability of the enzyme in the absence of substrate is shown in Figure 5a and b. The amylase and pullulanase activities were completely stable at 70 °C for 2 h. About 61 and 42% and 84 and 82% amylase pullulanase activities, remained after 2h at 80 and 90 °C, respectively. About 40 and 65% amylase and pullulanase activities, respectively, remained when the enzyme was incubated at 100 °C for 2 h. The thermal stability of the enzyme increased with the addition of substrate. About 63 and 76% of amylase and 70 and 87% of pullulanase activities were recorded when the enzyme was incubated at 100 °C in the presence of 1 and 3% starch up to 2 h. However, in the presence of 5% starch, both the enzyme activities were completely stable up to 2h at 100 °C (Figure 6a and b).
The thermal stability of amylase and pullulanase activities of the enzyme from different strains are not uniform (Hyun and Zeikus, 1985; Sata et al., 1989; Ara et al., 1992; Ganghofner et al., 1998; Ramesh et al., 1994; Swamy, 1994; Rudiger et al., 1995; Rama Mohan Reddy et al., 1998; Deweer et al., 2000; Gomes et al., 2003). In most of the cases, the thermal stability of the enzymes is determined in the absence of substrate and only in few cases both in the presence and absence of the substrate.

In the absence of the substrate, the amylopullulanase from Bacillus circulans F-2 (Sata et al., 1989) and Bacillus sp, KSM-1876 (Ara et al., 1992) is stable at 40 °C. The amylopullulanase from Thermoaerobacterium thermosaccharolyticum (Ganghofner et al., 1998) is stable at 60 °C for 8 h. The amylopullulanase from Thermoaerobacterium saccharolyticum B6A-RI (Ramesh et al., 1994) and Clostridium thermohydrodsulfuricum (Melasneimi, 1987) are stable at 65 °C with 80% activity for 1 h and 100% activity for 2 h, respectively. The amylopullulanase from Thermoaerobacter strain B6A (Saha et al., 1990) was stable at 70 °C. The amylopullulanase from Pyrococcus woesei (Rudiger et al., 1995) was stable at 90 °C for 4 h and 30 min.

The thermal stability of amylase and pullulanase in the absence of starch.

The thermal stability of amylase and pullulanase in the presence of starch.

The thermal stability of amylase and pullulanase produced by Clostridium thermohydrodsulfuricum, C. thermosulfurogenes SV9, C. thermosulfurogenes SV2 and Sulfolobus acidocaldarius (Hyun and Zeikus, 1985; Swamy, 1994; Rama Mohan Reddy et al., 1998; Deweer et al., 2000) were determined both in the presence and absence of the substrate. The presence of substrate increased the thermal stabilities of the above enzymes. The thermal stability of pullulanase by C. thermohydrodsulfuricum (Hyun and Zeikus, 1985) and amylase and pullulanase by C. thermosulfurogenes SV9 (Swamy, 1994) increased in the presence of 5% starch up to 85 and 75 °C, respectively. Rama Mohan Reddy et al., (1998) reported that the thermal stability of amylase and pullulanase by C. thermosulfurogenes SV2 was up to 80 °C for 2 h in the presence of 4% starch.
starch. Deweer et al., (2000) reported that the amylopullulanase from *Sulfolobus acidocaldarius* was stable up to 110 °C in presence of 0.5% w/v maltodextrin.

**pH activity and stability of the enzyme**

Both amylase and pullulanase activities showed a broad pH range (Figure 7). The pullulanase and amylase activities were more than 50 and 30% at pH values 4.0 and 8.0, respectively. The amylase and pullulanase activities increased from pH 4.0 and reached their maximum at pH 5.5 and maintained the maximum activity up to 6.0.

![Figure 7: Effect of pH on enzyme activity. Both amylase and pullulanase activities were assayed with 1% of respective substrates at 70 °C for 30 min with 0.1 M glycine hydrochloride (pH 2.0-3.0), sodium acetate (pH 4.0-5.5), sodium phosphate (pH 6.0-8.0) and glycine sodium (pH 9.0-10.0) buffers.](image)

Similar pH optima was observed for amylopolulnase from *Thermoanaerobacterium saccharolyticum* B6A-RI (Ramesh et al., 1994), *C. thermosulfurogenes* EMI (Spreinat and Antranikian, 1990), *P. woesei* (Rudiger et al., 1995), *P. furiosus* and *Thermococcus litoralis* (Brown and Kelly, 1993). The pH optima for amylopolulnase from *Thermoanaerobacterium thermosaccharolyticum* was 6.4 (Ganghofner et al., 1998). The amylopullulanase by *Sulfolobus acidocaldarius* DSM 639 has a very low pH optimum of 3.5 (Deweer et al., 2000), where as a high pH optimum of 8.5-10.0 was observed for the amylopullulanase from *Bacillus* sp. KSM-1876 (Ara et al., 1995).

Both amylase and pullulanase activities of the enzyme were completely stable in the pH range from 4.0 to 6.5. About 95 and 85% of pullulanase and amylase activities were stable at pH 3.0 and a steep fall in the stability was observed at pH 2.0 and above 7.0, respectively. The amylase activities were less stable than pullulanase activities in both acidic and alkaline pH ranges (Figure 8).

![Figure 8: Effect of pH on enzyme stability. Enzyme solutions were treated with 0.1 M glycine hydrochloride (pH 2.0-3.0), sodium acetate (pH 4.0-5.5), sodium phosphate (pH 6.0-8.0) and glycine sodium (pH 9.0-10.0) buffers and were incubated at 70 °C for 30 min before analyzing the residual activities.](image)

Effect of various metal ions / reagents on amylase and pullulanase activities of *C. thermosulfurogenes* SVM17

Metal ions have been known to stabilize and activate the enzymes (Swamy, 1994; Leveque et al., 2000; Vieille and Zeikus, 2001). The effect of metal ions and reagents on amylase and pullulanase activities of the strain SVM17 was examined at 5 and 10 mM concentrations of the metals (Table 2). These concentrations were selected based on the literature and on our own experience.

At 5 mM concentrations of Mg$^{2+}$, Ca$^{2+}$, Cu$^{2+}$, Fe$^{3+}$, Zn$^{2+}$, Hg$^{2+}$, EDTA, Cd$^{2+}$, Li$^{+}$, both amylase and pullulanase activities were inhibited. Amylase activity was strongly inhibited in the presence of Hg$^{2+}$ and complete inhibition was observed in the presence of Fe$^{3+}$. Apart from these, 0.1 mM urea, 5% ethanol, K$^{+}$, Cs$^{+}$, Li$^{+}$ and Mo$^{2+}$ also inhibited amylase activity. Whereas, pullulanase activity was inhibited in the presence of Ni$^{2+}$, Pb$^{2+}$ and Ce$^{3+}$. Complete inhibition of pullulanase activity was observed in the presence of Co$^{2+}$.

Many enzymes from α-amylase family, including amylopullulanases are Ca$^{2+}$ and Mg$^{2+}$ dependent. In contrast, the strain SVM17 showed slight inhibition of amylase and pullulanase activities in the presence of Ca$^{2+}$ and Mg$^{2+}$. Similar observations were made on amylase and pullulanase activities from *Thermoanaerobacterium* strain B6A (Saha et al., 1990), α-amylase activity from *B. licheniformis* (Umesh Kumar et al., 1990), *Bacillus* sp. L711 (Bernhardsdotter et al.,...
Table 2: Effect of metal ions / reagents on amylase and pullulanase activities of *C. thermosulfurogenes* SVM17.

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<tr>
<th>Metal ion / reagent</th>
<th>Concentration in mM</th>
<th>*Relative activity (%)</th>
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<td></td>
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2005) and pullulanase activity of *B. stearothermophilus* KP1064 (Suzuki and Imai, 1985). EDTA slightly inhibits, both the enzyme activities of strain SVM17 similar to the *Desulfurococcus mucosus* enzyme (Duffner et al., 2000) and *Bacillus* sp. A3-15 (Arikan, 2008) whereas, it strongly inhibited both amylase and pullulanase activities of *Bacillus* sp KSM 1378 (Ara et al., 1996), *C. thermohydrosulfuricum* (Saha et al., 1988), of 5mM concentration. Ag⁺ showed a decrease in stimulation and urea, ethanol and Mo²⁺ has no effect on the pullulanase activity.

The pullulanase activity of strain SVM17 was stimulated in the presence of Mn²⁺. Similar observations were made with the pullulanase activities of *T. hydrothermalis* (Gantalet and Duchiron, 1998), *Clostridium* isolate (Antranikian et al., 1987). From the above observations, it is clear that the pullulanase activity of the strain SVM17 is strongly inhibited by Ni²⁺, Zn²⁺, Co²⁺ and Pb²⁺ and the amylase activity was uninhibited and the hydrolysis of starch continued even in the presence of these inhibitors. The differential behavior of amylase and pullulanase activities towards some metal ions may be due to presence of two different active sites, one for amylase and other for pullulanase. Rudiger et al. (1995), reported that the pullulanase from *P. furiosus* has two active sites. Whereas, the pullulanase from *Thermoanaerobacterium* strain TOK 6-B1 and *C. thermohydrosulfuricum*, on the other hand, seem to possess only one active site (Plant et al., 1987; Mathupala et al., 1990).

**CONCLUSIONS**

In the present investigation, we have purified and characterized an enzyme which shows dual specificity for starch α-1,4 and α-1,6 glucocidic linkage and produced maltose as major end product of starch hydrolysis. The ability of this enzyme withstanding a temperature of 100°C over a broad pH range suggests as catalyst in a one step liquefaction-saccharification process for the production of high maltose syrups.

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