Malaysian Journal of Microbiology, Vol 7(2) 2011, pp. 97-106

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

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Received 3 September 2010; received in revised form 15 January 2011; accepted 15 January 2011

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, Mn^{2+} and Ag^{+} strongly stimulated both amylase and pullulanase activities, where as Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{3+}, Zn^{2+}, Hg^{2+}, EDTA, Cd^{2+} and Li^{2+} inhibited both amylase and pullulanase activities. When the concentration of metal ions was increased from 5 to10 mM, a further increase in amylase activity was observed in the presence of Ni^{2+}, Mn^{2+} and Co^{2+}. Where as substantial decrease was observed at 10 mM concentration of Ag^{+}, Pb^{2+} and Ca^{2+}.

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

INTRODUCTION

Maltose 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 thermoactivity 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 enzyme 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

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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 × *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 × *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 x 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 mL of 3,5-dinitrosalicylic acid (Miller, 1959). A separate blank was set up for each sample to correct the non-enzymatic release of sugars. One unit of amylase or pullulanase is defined as the amount of enzyme which released 1 µmole of reducing sugars (as glucose equivalents) per min under the standard assay conditions described above.

Determination of protein

Protein content was determined by the method of Lowry *et al*. (1951) with bovine serum albumin as standard. Protein content in the column eluates was routinely followed by absorbance at 280 nm.

Determination of molecular weight

SDS-Slab gel electrophoresis was carried out at room temperature (26 ± 2 °C) according to the method described by LaemmLi (1970) and Blum *et al*. (1987). The standard markers of medium range (14-116 kDa) were used.

Identification of hydrolysis products

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, maltotriose 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 R_f 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 on 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 pullanase 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 and 38.20% 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.

Procedure	Total protein (mg)	Total activity (U)		Specific activity (U/mg of protein)		Purification factor		Yield (%)	
		Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase
Culture supernatant	3122	951	468	0.31	0.15	1	1	100	100
Ammonium sulfate precipitation and dialysis	349	363	263	1.04	0.75	3.4	5	38.2	56.2
Gel filtration using sephadex G200									
I	26.1	295.5	183.82	11.32	7.04	33.29	46.95	31.02	39.28
II	7.4	102	76	13.78	10.31	44.45	68.73	10.73	16.28

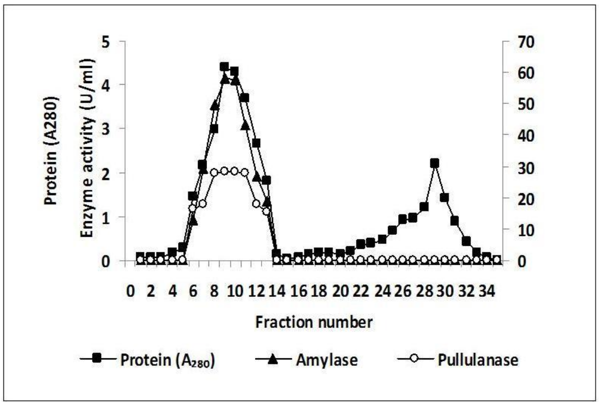

Figure 1: Elution profile of amylase and pullulanase on sephadex G-200.

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).

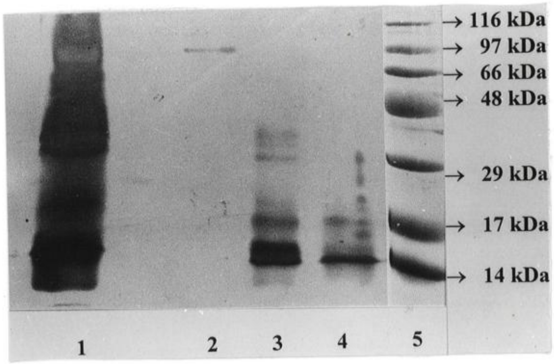

Figure 2: Analysis of purified amylolytic enzyme on SDS-PAGE, gel stained with silver staining. Lane 1 indicates the complete protein profile of extra cellular amylolytic enzyme. Lane 2 indicates the purified amylolytic enzyme, Lane 3 and 4 indicates different fractions eluted by column chromatography 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 amylopullulanase (Saha and Zeikus, 1987).

Molecular weight of purified amylopullulanase

The molecular weight of purified enzyme determined by SDS-PAGE was 97 kDa (Figure 2). The molecular weight of amylopullulanase from different organisms ranged between 40 and 450 kDa. The molecular weights of amylopullulanase 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 *Sulfolobus acidocaldarious* (Deweer *et al*., 2000) were 220, 83, 110, 90, 90, 102, 210 and 95 kDa, respectively. The molecular weight (97 kDa) of amylopullulanase by *C. thermosulfurogenes* SVM17 is with in the range and closer to that of *C. thermosulfurogenes* EMI amylopullulanase. The amylopullulanase produced by *Thermococcus profundus* had a very low molecular weight of 43 kDa (Kwak *et al*., 1998), where as the enzyme from *Thermoanaerobacter* strain B6A (Saha *et al*., 1990), has a high molecular weight of 450 kDa. Most of the amylopullulanase 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.

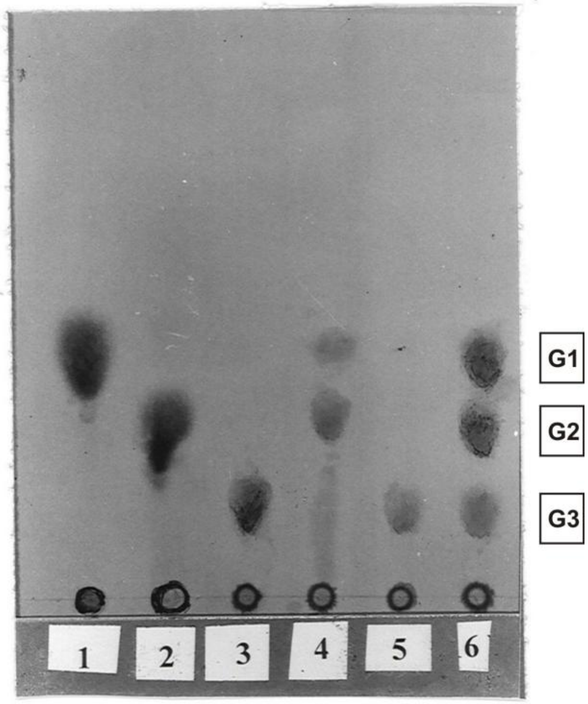

Figure 3: TLC of the hydrolysed products of starch and pullulan formed by the action of purified SVM17 amylolytic enzyme. Lane 1, 2 and 3 indicates standard glucose, maltose and maltotriose, respectively. Lane 4 shows the hydrolytic products of starch, lane 5 shows the hydrolytic product of pullulan 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.

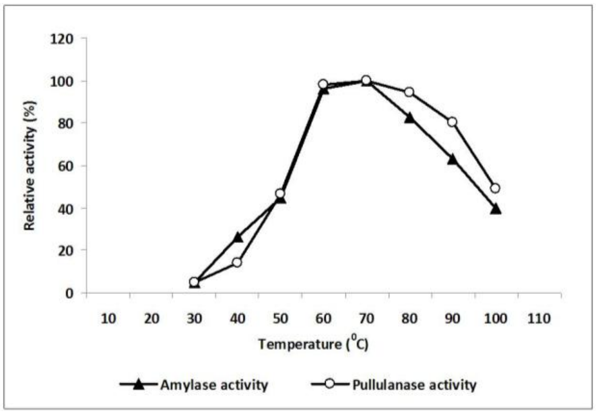

Figure 4: Effect of temperature on enzyme activity.

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).

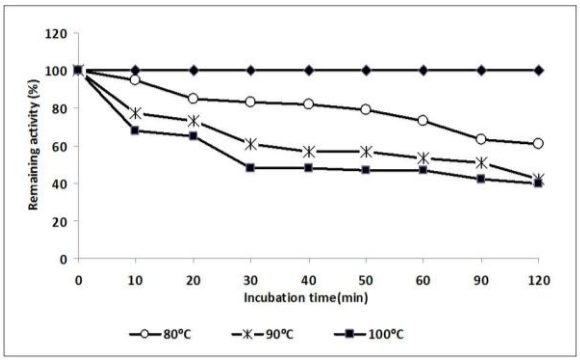

Figure 5a: Thermostability of amylase in the absence of starch.

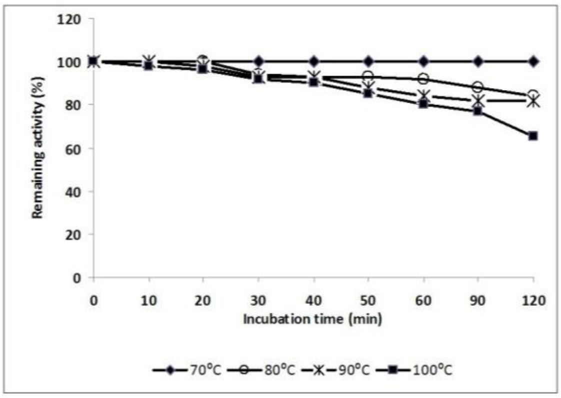

Figure 5b: Thermostability of pullulanase in the absence of starch.

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; Ganghofnor *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 ^{o}C. The amylopullulanase from *Thermoanaerobacterium thermosaccharolyticum* (Ganghofner *et al*., 1998) is stable at 60 °C for 8 h. The amylopullulanase from *Thermoanaerobacterium saccharolyticum* B6A-RI (Ramesh *et al*., 1994) and *Clostridium thermohydrosulfuricum* (Melasneimi, 1987) are stable at 65 °C with 80% activity for 1h and 100% activity for 2 h, respectively. The amylopullulanase from *Thermoanaerobacter* 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.

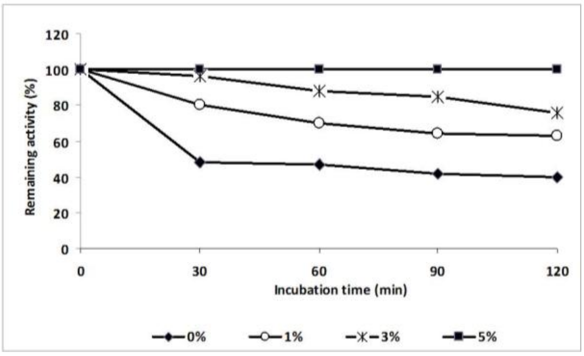

Figure 6a: Thermostability of amylase in the presence of starch at 100 °C. Reaction mixture containing enzyme solution, 0.1M acetate buffer (pH 5.5) and indicated amount of soluble starch were incubated at 100 °C and sample were withdraw at various time intervals as shown and the remaining activities were assayed under standard assay condition.

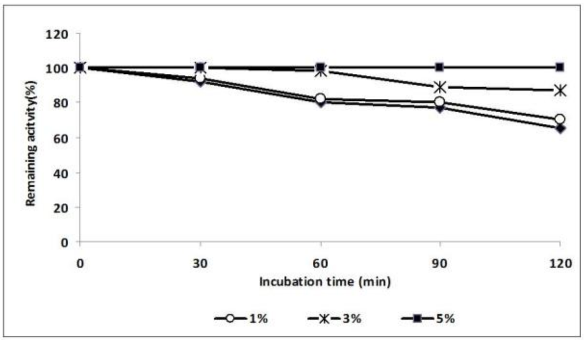

Figure 6b: Thermostability of pullulanase in the presence of starch at 100 °C. Reaction mixture containing enzyme solution, 0.1 M acetate buffer (pH 5.5) and indicated amount of soluble starch were incubated at 100 °C and sample were withdraw at various time intervals as shown and the remaining activities were assayed under standard assay condition.

The thermal stabilities of amylolytic enzymes produced by *Clostridium thermohydrosulfuricum, 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. thermohydrosulfuricum* (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. Deweer *et al*., (2000) reported that the amylopullulanase from *Sulfolobus acidocaldarious* 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.

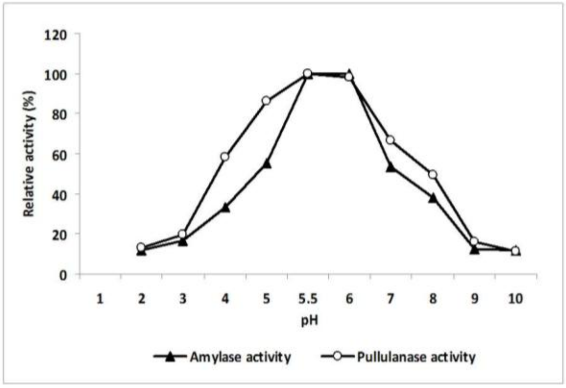

Figure 7: Effect of pH on enzyme activity. Both amylase and pullulanase activities were assayed with 1 % of respective substrates at 70 ^{o}C for 30 min with 0.1 M glycine hydrochloride (pH 2.0-3.0), sodium acetate (pH 4.0-5.5), sodium phosphate (pH 6.0-8.0) and glycine sodium (pH 9.0-10.0) buffers.

Similar pH optima was observed for amylopullulanase 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 amylopullulanase 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).

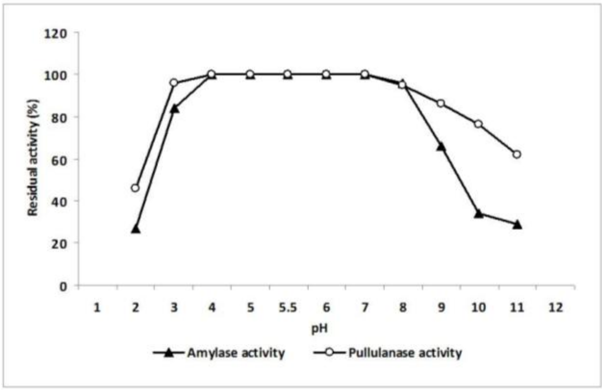

Figure 8: Effect of pH on enzyme stability. Enzyme solutions were treated with 0.1 M glycine hydrochloride (pH 2.0-3.0), sodium acetate (pH 4.0-5.5), sodium phosphate (pH 6.0-8.0) and glycine sodium (pH 9.0-10.0) buffers and were incubated at 70 °C for 30 min before analyzing the residual activities.

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^{2+}, 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^{2+}. Apart from these, 0.1 mM urea, 5% ethanol, K^{+}, Cs^{2+}, Li^{+} and Mo^{2+} also inhibited amylase activity. Whereas, pullulanase activity was inhibited in the presence of Ni^{2+}, Pb^{2+} and Ce^{2+}. 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. LI711 (Bernhardsdotter *et al*.,

Table 2: Effect of metal ions / reagents on amylase and pullulanase activities of *C. thermosulfurogenes* SVM17.

Metal ion / reagent	Concentration in mM	*Relative activity (%)	
		Pullulanase activity	Amylase activity
None		100	100
$MgCl_2$	5	47	65
	10	10	28
$CaCl_2$	5	88	65
	10	84	59
$CuSO_4$	5	45	53
	10	92	41
$FeCl_3$	5	23	62
	10	43	0
$FeSO_4$	5	157	0
	10	225	44
$ZnCl_2$	5	41	87
	10	39	114
$HgCl_2$	5	67	27
	10	43	0
Urea	0.1	112	76
	1.0	108	89
$K_3Fe(CN)_6$	0.1	110	81
	1.0	86	116
EDTA	5	43	60
	10	41	70
Ethanol	5%	102	52
	10%	102	93

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 Mo^{2+} has no effect on the pullulanase activity.

The pullulanase activity of strain SVM17 was stimulated in the presence of Mn^{2+}. 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 Ni^{2+}, Zn^{2+}, Co^{2+} and Pb^{2+} 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.

ACKNOWLEDGEMENTS

The authors acknowledge Council of Scientific and Industrial Research (CSIR), New Delhi, India, for providing funds. One of the authors (S. Mrudula) thanks the CSIR for award of senior research fellowship during the course of this investigation.

REFERENCES

Antranikian, G., Herzberg, C. and Gottschalk. G. (1987). Production of thermostable α-amylase, pullulanase, and α-glucosidase in continuous culture by a new *Clostridium* isolate, *Applied and Environonmental Microbiol*ogy **53, 1668-1673.**

Ara, K., Igarashi, K., Shigihara, H., Sawada, K., Kobayashi, T. and Ito, S. (1996). Separation of functional domains for the α-1,4 and α-1,6 hydrolytic activities of *Bacillus* amylopullulanase by limited proteolysis with papain. *Bioscience, Biotechnology and Biochemistry* **60, 634-639.**

Ara, K., Igarashi, K., Saeki, K., Kawai, S. and Ito, S. (1992). Purification and some properties of an alkaline pullulanase from alkaliphilic *Bacillus* sp. KSM–1876. *Bioscience, Biotechnology and Biochemistry* **56, 62-65.**

Ara, K., Igarashi, K., Saeki, K. and Ito, S. (1995). An alkaline amylopullulanase from alkaliphilic *Bacillus* sp. KSM-1378: Kinetic evidence for two independent active sites for the α-1,4 and α-1,6 hydrolytic reactions. *Bioscience, Biotechnology and Biochemistry* **59, 662-666.**

Arikan, B. (2008). Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic *Bacillus* sp. Isolate A3-15. *Bioresource Technol*ogy **99, 3071-3076.**

Bernhardsdotter, E. C. M. J., Joseph, D. N. G., Garriott, O. K. and Pusey, M. L. (2005). Enzymatic properties of an alkaline chelator-resistant α-amylase from an alkaliphilic *Bacillus* sp. isolate L1711. *Process Biochemistry* **40, 2401-2408.**

Blum, I., Beier, H. and Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8, 93-99.**

Brown, S. H. and Kelly, R. M. (1993). Characterization of amylolytic enzymes, having both α-1,4 and α-1,6 hydrolytic activity, from the thermophilic archea *Pyrococcus furiosus* and *Thermococcus litoralis*. *Applied and Environmental Microbiol*ogy **59, 2614-2621.**

Deweer, P., and Amory, A. (2000). Acid-stable and thermostable enzymes derived from *Sulfolobus* species. USP No.6,100,073.

Dong, G., Vieille, C. and Zeikus, J. G. (1997). Cloning, sequencing and expression of the gene encoding amylopullulanase from *Pyrococcus furiosus* and biochemical characterization of the recombinant enzyme. *Applied and Environmental Microbiol*ogy **63, 3577-3584**.

Duffner, F., Bertoldo, C., Andersen, J. T., Wagner, K. and Antranikian, G. (2000). A new thermoactive pullulanase from *Desulfurococcus mucosus*: Cloning, sequencing, purification and characterization of the recombinent enzyme after expression in *Bacillus subtilis*. *Journal of Bacteriol*ogy **182, 6331-6338**.

Fogarty, W. M. and Kelly, C. T. (1994). Extracellular maltotetrose forming amylase of *Pseudomonas* sp. IMD 353. *Biotechnology Letters* **16, 473-478.**

Ganghofner, D., Kellermann, J., Staudenbauer, L.W. and Bronnenmeier, K. (1998). Purification and properties of an amylopullulanase, a glucoamylase, and an α-glucosidase in the amylolytic enzyme system of *Thermoanaerobacterium thermosaccharolyticum*. *Bioscience Biotechnology and Biochem*istry **62, 302-308.**

Gantelet, H. and Duchiron, F. (1998). Purification and properties of a thermoactive and thermostable pullulanase from *Thermococcus hydrothermalis*. a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Applied Microbiology and Biotechnol*ogy **49, 770-777.**

Gomes, J., Gomes, I. and Steiner, W. (2003). Highly thermostable amylase and pullulanase of the extreme thermophilic eubacterium *Rhodothermus marinus*: Production and partial characterization. *Bioresource Technology* **90, 207-214.**

Haki, G. D. and Rakshit, S. K. (2003). Developments in industrially important thermostable enzymes: A review. *Bioresource Technology* **89, 17-34.**

Hyun, H. H. and Zeikus, J. G. (1985). General and biochemical characterization of thermostable extracellular β-amylase from *Clostridium thermosulfurogenes*. *Applied and Environmental Microbiology* **49**, **1162-1167.**

Kim, T. U., Gu, B. G., Jeong, J. Y., Byun, S. M. and Shin, Y. C. (1995). Purification and characterization of maltotetraose forming alkaline *Bacillus* strain GM 8901. *Applied and Environmental Microbiology* **61, 3105-3112.**

Kwak, Y., Akeba, T. and Kudo, T. (1998). Purification and characterization of α-amylase from hyperthermophilic archaeon *Thermococcus profundus*, which hydrolyses both α-1,4 and α-1,6-glucosidic linkages. *Journal of Fermentation and Bioengineering* **86, 363-367.**

LaemmLi, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227, 680-685.**

Lin, L., Chyau,C. C. and Hsu, W. H. (1998). Production and properties of a raw starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. TS-23. *Biotechnology and Applied Biochemistry* **28, 61-68.**

Leveque, E., Janecek, S., Haye, B. and Belarbi, A. (2000). Thermophilic archael amylolytic enzymes. *Enzyme and Microbial Technology* **26, 3-14.**

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with folin phenol reagent. *Journal of Biological Chemistry* **193, 265-275.**

Mathupala, S., Saha, B. C. and Zeikus, J. G. (1990). Substrate competition and specificity at the active site of amylopullulanase from *Clostridium thermohydrosulfuricum*. *Biochemical and Biophysical Research Communications* **166**, **126-132.**

Melasniemi, H. (1987). Characterization of α-amylase and pullulanase activities of *Clostridium thermohydrosulfuricum*. *Biochemical Journal* **246, 193-197.**

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry* **31, 426-428.**

Mrudula, S., Gopal Reddy and Seenayya,G. (2010). Screening of medium components for production of thermostable amylopullulanase by *Clostridium thermosulfurogenes* SVM 17 in submerged fermentation using Plackett-Burman Design. *Asian Journal of Microbiology Biotechnology and Environmental Sciences* **3, 4-9.**

Mrudula, S. (2010). Optimization of thermostable amylopullulanase production in solid state fermentation by *Clostridium thermosulfurogenes* SVM 17 through Plackett-Burman and response surface methodological approaches. *Malaysian Journal of Microbiology* **6(2), 181-195.**

Mrudula, S., Gopal Reddy and Seenayya,G. (2011). Effect of process paprameters on production of thermostable amylopullulanase by *Clostridium thermosulfurogenes* SVM 17 under solid state fermentation. *Malaysian Journal of Microbiology* **7(1), 19-25.**

Plant, A. R., Morgan, H. W.and Daniel, R. M. (1986). A highly stable pullulanase from *Thermus aquaticus* YT-1. *Enzyme and Microbial Technology* **668-672**.

Plant, A. R., Clemens, R. M., Daniel, R. M. and Morgan, H. W. (1987). Purification and preliminary characterization of an extra cellular pullulanase from *Thermoanaerobium* Tok6-BI. *Applied Microbiology and Biotechnology* **26, 427-433.**

Ramesh, M. V., Podkovyrov, S. M., Lowe S. E. and Zeikus, J. G. (1994). Cloning and sequencing of the *Thermoanaerobaterium saccharolyticum* B6A-RI apu gene and purification of the amylopullulanase from *Escherichia coli. Applied and Environmental Microbiology* **60, 94-101.**

Rama Mohan Reddy, P., Swamy, M. V. and Seenayya, G. (1998). Purification and characterization of thermostable β-amylase and pullulanase from high-yielding *Clostridium thermosulfurogenes* SV2. *World Journal of Microbiology and Biotechnology* **14, 89-94**.

Rudiger, A., Jorgensen, P.L. and Antranikian, G. (1995). Isolation and characterization of a heat stable pullulanase from the hyperthermophilic archaeon *Pyrococcus woesei* after cloning and expression of its gene in *Escherichia coli*. *Applied and Environmental Microbiology* **61**, **567-575.**

Saha, B. C.and Zeikus, J. G. (1987). Biotechnology of maltose syrup production. *Process Biochemistry* **22, 78-82.**

Saha, B. C., Mathupala, S. P. and Zeikus, J. G. (1988). Purification and characterization of a highly thermostable novel pullulanase from *Clostridium thermohydrosulfuricum*. *Biochemical Journal* **252, 343-348.**

Saha, B. C., Lamed, R. L., Lee, C. Y. Mathupala, S. P. and Zeikus, J. G. (1990). Characterization of an endo-acting amylopullulanase from *Thermoanaerobacter* strain B6A. *Applied and Environmental Microbiology* **56, 881-886.**

Sata, H., Umeda, M., Kim, C. H., Taniguchi, H. and Maruyama, Y. (1989). Amylase-Pullulanase enzyme produced by *B. Circulans* F-2. *Biochimica et Biophysica Acta* **991, 388-394.**

Saxena, R. K., Dutt, K., Agarwal, L. and Nayyar, P. (2007). A highly thermostable and alkaline amylase from a *Bacillus* sp. PN5. *Bioresource Technology* **98, 260-265.**

Shafiei, M., Zjaee, A. A. and Amoozegar, M. A. (2010). Purification and biochemical characterization of a novel SDS and surfactant stable raw starch digesting and halophilic α-amylase from moderately halophilic bacterium *Nesterenkonia sp*. strain F. *Process Biochemistry* **45, 694-699.**

Shen, G. J., Saha, B. C., Lee, Y. E., Bhatnagar, L and Zeikus, J. G. (1988). Purification and characterization of a novel thermostable β-amylase from *Clostridium thermosulfurogenes. Biochemical Journal* **254**, **835-840.**

Sivaramakrishnan,S., Gangadharan, D., Madhavan Nampoothiri, K., Soccol, C. R. and Pandey, A. (**2006**). α-amylases from microbial source-An overview on recent developments. *Food Technology and Biotechnology* **44, 173-184.**

Soni, S. K., Sodhi, H. K., Sharma, K., Gupta, J. K. (2005). Production of thermostable α-amylase from *Bacillus sp*. PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production. *Process Biochemistry* **40, 525-534.**

Spreinat, A. and Antranikian, G. (1990). Purification and properties of a thermostable pullulanase from *Clostridium thermosulfurogenes* EM1 which hydrolyses both α-1,6 and α-1,4 glycosidic Linkages. *Applied Microbiology and Biotechnology* **33, 511-518.**

Suzuki, Y. and Imai, T. (1985). *Bacillus stearothermophilus* KP 1064 Pullulan hydrolase, its assignment to a unique type of maltogenic α-amylase but neither pullulanase nor isopullulanase. *Applied Microbiology and Biotechnology* **21, 20-26.**

Swamy, M. V. (1994). A study into maltose and maltooligosaccharides producing thermostable amylases from *Clostridium thermosulfurogenes* SV9, Ph.D. Thesis, Osmania University, Hyderabad, India.

Swamy, M. V. and Seenayya, G. (1996a). *Clostridium thermosulfurogenes* SV9-A thermophilic amylases producer. *Indian Journal of Microbiology* **36, 181-184.**

Swamy, M. V. and Seenayya, G. (1996b). Thermostable pullulanase and α-amylase from *Clostridium thermosulfurogenes* SV9: Optimization of culture conditions for enzymes production. *Process Biochemistry* **31, 157-162.**

Umesh Kumar, S., Rehana, F. and Nand, K. (1990). Production of an extracellular thermostable calcium-inhibited α-amylase by *Bacillus licheniformis* MY 10. *Enzyme and Microbial Technology* **12, 20-26.**

Vieille, C and Zeikus, J. G. (2001). Hyperthermophilic enzymes: Sources, uses, and Molecular mechanisms for thermostability. *Microbiology and Molecular Biology Reviews* **65, 1-43.**

Zareian, S., Khajeh, K., Ranjbar, B., Dabirmanesh, B., Ghollasi, M., Mollania, N. (2010). Purification and characterization of a novel amylopullulanase that converts pullulan to glucose, maltose and maltotriose and starch to glucose and maltose. *Enzyme and Microbial Technology*. **46, 57-63.**