

Effect of substrate and culture conditions on the production of amylase and pullulanase by thermophilic *Clostridium thermosulfurogenes* SVM17 in solid state fermentation

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ABSTRACT

The endo acting enzyme with dual specificity towards α -1,4- and α -1,6-glycosidic linkages are named as amylopullulanase. The production of extracellular thermostable amylopullulanase by *Clostridium thermosulfurogenes* SVM17 was investigated in solid state fermentation (SSF). Coarse type wheat bran was found to be the best substrate among ten easily available complex organic substrates evaluated. The production of enzyme reached a peak in 72 h. A high level of enzyme was produced in wheat bran moistened with PYE medium with a moisture content of 73 %. The optimum temperature and pH for amylopullulanase production was 60 °C and 7.5, respectively. An inoculum size of 20 % resulted in maximum production of amylopullulanase. Under the optimum conditions the strain showed a maximum of 17,227 and 21,526 U of amylase and pullulanase activity, respectively per kilogram of bacterial bran (BB). The enzyme production was high in SSF than that in SmF. The use of SSF for the production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM17 could, therefore led to reduction in the overall cost of enzyme production.

Keywords: *Clostridium thermosulfurogenes*, amylase activity, pullulanase activity, process parameters, solid state fermentation.

INTRODUCTION

Maltose and maltooligosaccharides find wide range of applications in food, beverage, pharmaceutical and chemical industries (Fogarty and Kelly, 1990). They are produced by hydrolysis of starch using amylases from higher plants, certain mesophilic bacteria and fungi. The majority of amylases reported so far are optimally active at moderate temperatures (Haki and Rakshit, 2003). Thermal stability of amylolytic enzymes were reported in the range of 60-90 °C (Hyun and Zeikus, 1985a; b; Saha *et al.*, 1990; Ramesh *et al.*, 1994; Swamy *et al.*, 1994; Chung *et al.*, 1995; Busch and Stutzenberger, 1997; Ganghofner *et al.*, 1998; Reddy *et al.*, 1998; Gessesse and Mamo, 1999; Gomes *et al.*, 2003; Sodhi *et al.*, 2005). Therefore, a high value is placed on extreme thermostability and thermoactivity of the enzymes. Thermoanaerobic bacteria are of considerable interest as producers of thermostable amylolytic enzymes (Antranikian, 1990). In this direction, anaerobic and thermophilic bacteria that secrete amylases producing maltose and malto oligosaccharides were isolated in our laboratory (Swamy and Seenayya, 1996a; Mrudula, 2010). These strains (Reddy *et al.*, 1998; Mrudula, 2010; Swamy and Seenayya, 1996a; b) were screened for the production of maltose and maltooligosaccharides and the

strain, *Clostridium thermosulfurogenes* SVM17, which produced increased yields of enzyme having properties of both amylase and pullulanase and stable at 100 °C was selected for the detailed study (Mrudula, 2001). This type of endo acting enzyme with dual specificity towards α -1,4- and α -1,6-glycosidic linkages has been named as amylopullulanase (Saha and Zeikus, 1989; Saha *et al.*, 1990; Kwak *et al.*, 1998). Since the enzyme was stable at higher temperatures and able to hydrolyze α -1,4-bonds in starch and amylose and α -1,6-bonds in pullulan and branched chain polysaccharides, therefore, has a potential application for enhancement of the starch saccharification process in industry (Ramesh *et al.*, 1994). The amylopullulanases are reported from number of thermophilic bacterial species, such as *Bacillus* sp. strain 3183 (Saha *et al.*, 1989), *Thermus aquaticus* (Plant *et al.*, 1986), *Thermus* sp. strain AMD33 (Nakamura *et al.*, 1989), *Clostridium thermohydrosulfuricum* (Melasniemi, 1987; 1988; Saha *et al.*, 1988), *Clostridium thermosulfurigen* EM1 (Spreinat and Antranikian, 1990), *Thermoanaerobium* sp. strain Tok 6-B1 (Plant *et al.*, 1987), *Thermoanaerobacter* sp. strain B6A (Saha *et al.*, 1990), *Lactobacillus plantarum* L137 (Kim *et al.*, 2008), *Geobacillus stearothermophilus* (Zareian *et al.*, 2010).

In order to improve the yield of an enzyme, a suitable cultivation system is required. Most of the amylolytic

enzymes so far reported have been studied in submerged fermentation (SmF). A very few reports on these enzymes have been reported in solid-state fermentation (SSF). α -Amylase by *Bacillus megaterium* 16M (Ramesh and Lonsane, 1987), *B. subtilis* (Baysal *et al.*, 2003) *B. cereus* MTCC 1305 (Anto *et al.*, 2006) *B. licheniformis* M27 (Ramesh and Lonsane., 1990), *B. amyloliquefaciens* (Gangadharan *et al.*, 2006) and *B. coagulans* (Babu and Satyanarayana, 1995), β -amylase and pullulanase by *Clostridium thermosulfurogenes* SV2 (Reddy *et al.*, 2000). No reports are available in the literature on the production of thermostable amylopullulanase by SSF process. In the present study, the influence of various process parameters on production of thermostable amylopullulanase by SSF are reported.

MATERIALS AND METHODS

Bacterial strain

The bacterial strain used in the present study was isolated in our laboratory (Swamy and Seenayya, 1996a) and identified as *Clostridium thermosulfurogenes* SVM17.

Solid-state fermentation

Approximately 10 g of wheat bran having uniform particle size was taken in 120 mL serum vial moistened with 1:1.5 w/v PYE medium (Swamy and Seenayya, 1996b) containing (g/L): NH_4Cl , 1.0; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2; KH_2PO_4 , 0.3; $\text{NaHPO}_4 \cdot 7\text{H}_2\text{O}$, 2.0; peptone, 10.0; yeast extract, 3.0; 2.5% (w/v) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 mL; trace mineral solution, 10 mL; vitamin solution, 5 mL; resazurin, 0.002.

The trace mineral solution contained (g/L): Nitritotriacetic acid, 12.8 (neutralized to pH 6.5 with KOH); $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.1; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.16; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1; ZnCl_2 , 0.1; CuCl_2 , 0.02; H_3BO_3 , 0.01; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.01; NaCl , 1.0; $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$, 0.026; Na_2SeO_3 , 0.02. The vitamin solution contained ($\mu\text{g/L}$): biotin, 1.0; cyanocobalamine, 2.0; pyredoxinhydrochloride, 8.0; *p*-aminobenzoic acid, 4.0. The medium was flushed with nitrogen gas to create anaerobic conditions. The sealed vials were sterilized for 15 min at 15 psi-121 °C. After cooling to room temperature (28 ± 2 °C), a 2% (v/v) of 2.5% (w/v) Na_2S solution was added to maintain further reduced conditions followed by 2 mL of 24 h old inoculum. The contents in the vials were mixed thoroughly and incubated at 60 °C in horizontal position. The contents in the vials were periodically mixed by gentle shaking and accumulated gases were removed using a sterile needle. At the end of incubation (72 h), vials were taken out and enzyme from each vial was extracted with 0.1 M sodium acetate buffer (pH 5.5) at 1:5 w/v ratio 28 ± 2 °C with a contact time of 1 h at an agitation speed of 150 rpm on a rotary shaker. Extracts were clarified by squeezing through dampened cheese cloth (Ramesh and Lonsane, 1990), followed by centrifugation at 8000 rpm for 20 min at 4 °C, and the supernatant was used as source of extracellular enzyme.

Enzyme assay

Various methods of enzyme assays have been reported in literature. Recently Gupta *et al.*, (2003) and Xiao *et al.*, (2006) have reviewed the methodologies on enzyme assays. However the basic method of Miller (1959) is the most widely adapted for assay of amylase, pullulanase activities. In the present study amylase and pullulanase activities were assayed by measuring the reducing sugar released from the action of starch and pullulan respectively.

Amylase activity

The reaction mixture (3 mL) composed of 0.5 mL soluble starch (1% w/v) and 0.5 mL of appropriately diluted enzyme source in 2 mL of sodium acetate buffer (0.1M, pH 5.5). After incubation at 70 °C for 30 min, the reaction was stopped by cooling the tubes in an ice bath and reducing sugar released by enzymatic hydrolysis of starch was determined by addition of 1 mL of 3,5-dinitrosalicylic acid (Miller, 1959; Ramesh *et al.*, 1994; Okolo *et al.*, 1995). Color development was read at 540nm using UV visible Spectrometer (Shimadzu UV-VIS 260A). A separate blank was set up for each sample to correct the non enzymatic release of sugars. One unit of amylase is defined as the amount of enzyme which released one micro mole of reducing sugars as glucose per min under standard assay conditions.

Pullulanase activity

The standard assay procedure used for measurement of pullulanase activity consisted of a reaction mixture (3 mL) composed of 0.5 mL pullulan (1% w/v) and 0.5 mL of appropriately diluted enzyme source in 2 mL of sodium acetate buffer (0.1 M, pH 5.5). After incubation at 70 °C for 30 min, the reaction was stopped by cooling the tubes in an ice bath and reducing sugar released by enzymatic hydrolysis of pullulan was determined by addition of 1 mL of 3,5-dinitrosalicylic acid (Miller, 1959; Ramesh *et al.*, 1994; Duffner *et al.*, 2000). Color development was read at 540 nm using UV visible Spectrometer (Shimadzu UV-VIS 260A). A separate blank was set up for each sample to correct the non enzymatic release of sugars.

One unit of pullulanase is defined as the amount of enzyme which released one micro mole of reducing sugars as glucose per min under standard assay conditions.

Effect of various complex organic solid substrates on enzyme production

The strain SVM17 was grown in 120 mL serum vials containing 10 g each of various solid substrates (de oiled cakes of coconut and ground nut, coarse type of brans of pulses such as red gram, black gram, bengal gram, green gram, coarse as well as fine types of rice and wheat brans) and moistened with 1:1.5 PYE medium, sterilized, inoculated and incubated at 60 °C for 24, 48 and 72 h,

respectively. At the end of incubation, the enzyme was extracted and assayed.

Effect of incubation temperature on enzyme production

Effect of incubation temperature on production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM17 was studied by incubating the serum vials containing SSF medium at different temperatures ranging between 30 and 70 °C.

Effect of initial pH on enzyme production

The procedure followed for adjusting the pH was based on methodology of Suresh and Chandrasekaran (1999). The effect of initial pH of the medium on the yield of amylopullulanase was studied by growing the culture at different initial pH between 4.0 and 9.0. The pH of the PYE medium (moistening agent) was adjusted with 1 N H₂SO₄ or 1 N NaOH before using it for moistening wheat bran.

Effect of moisture content on enzyme production

The influence of initial moisture level on enzyme production was studied by varying the ratio (w/v) of wheat bran to PYE medium (moistening agent) between 0.5 and 2.5 (i.e., 30 and 75% moisture content).

Effect of moistening agents on amylopullulanase production

The effect of moistening agents such as tap water, distilled water and PYE medium on enzyme production was studied.

Inoculum size

The SSF medium was inoculated with 5, 10, 15, 20 and 25% of exponentially grown culture and incubated at 60 °C for 72 h. The enzyme was assayed at the end of incubation period.

RESULTS AND DISCUSSION

It is clear from Figures 1 and 2 that the incubation time required for maximum production of enzyme varied on the type of substrate used. Among the different solid substrates screened, coarse type of wheat bran supported maximum yields of both amylase and pullulanase at 72 h, followed by fine type of wheat bran, rice bran, groundnut cake, black gram bran in the decreasing order. The peak production of enzyme yields was observed at 48 h on red gram bran and coarse type of rice bran. The enzyme yields of both amylase and pullulanase decreased gradually after 24 h when grown on coconut cake, brans of green gram and bengal gram. Thus, wheat bran was found to be a better substrate. In SSF, the substrate supplies the nutrients for growth of the microorganisms

and also provides anchorage to the cells (Lonsane *et al.*, 1985; Babu and Satyanarayana, 1996). Presence of sufficient nutrients and non sticky property even in moist conditions and there by providing large surface area (Babu and Satyanarayana, 1995; Feniksova *et al.*, 1960) may be responsible for the wheat bran to be more suitable.

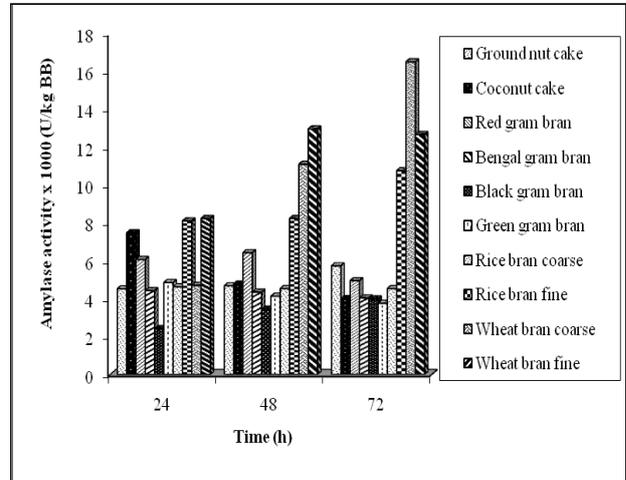


Figure 1: Effect of various complex organic solid substrates on yields of amylase by *C. thermosulfurogenes* SVM17 under solid state fermentation. The values reported in the figure are the average values of two experiments in triplicates carried out on different occasions

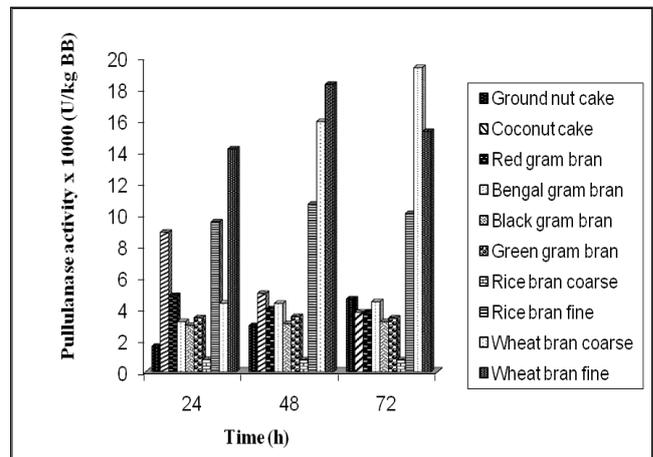


Figure 2: Effect of various complex organic solid substrates on yields of pullulanase by *C. thermosulfurogenes* SVM17 under solid state fermentation. The values reported in the figure are the average values of two experiments in triplicates carried out on different occasions

The maximum yield of the enzyme reached in 72 h and further increase in the incubation time did not influence on the enzyme yields (Figure 3). Considerable amounts of enzyme yield was recorded over a range of pH and temperatures (Figures 4 and 5) and the optimum pH and temperature required for the production of the enzyme were 7.5 and 60 °C, respectively. The maximum growth and enzyme production of *C. thermosulfurogenes* SVM17 occurred at a temperature of 60 °C. Similar observations were reported for β -amylase and pullulanase production by *C. thermosulfurogenes* SV2 (Reddy *et al.*, 2000). For α -amylase production by *B. cereus* MTCC 1305, 55 °C was found optimum (Anto *et al.*, 2006).

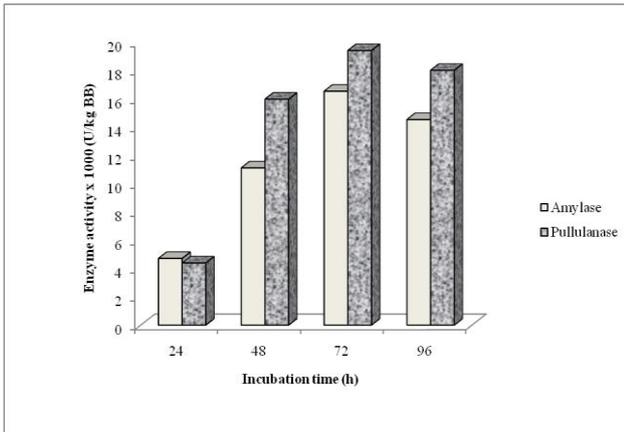


Figure 3: Effect of incubation time on amylopullulanase production by *C. thermosulfurogenes* SVM17 under solid state fermentation. The values reported in the figure are the average values of two experiments in triplicates carried out on different occasions

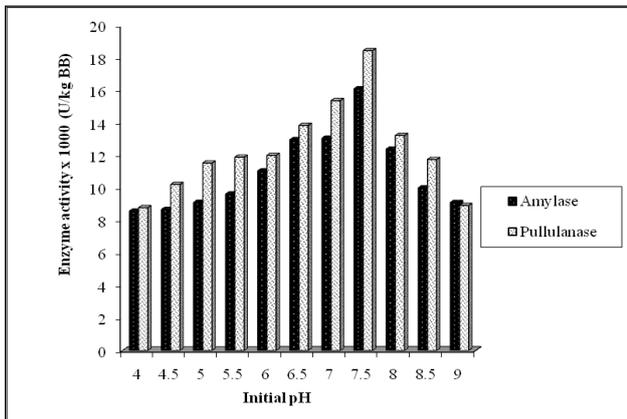


Figure 4: Effect of initial pH on amylopullulanase production by *C. thermosulfurogenes* SVM17 under solid state fermentation. The values reported in the figure are the average values of two experiments in triplicates carried out on different occasions

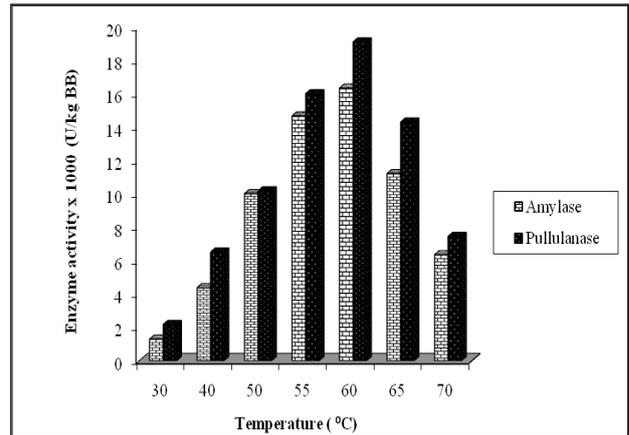


Figure 5: Effect of incubation temperature on amylopullulanase production by *C. thermosulfurogenes* SVM17 under solid state fermentation. The values reported in the figure are the average values of two experiments in triplicates carried out on different occasions

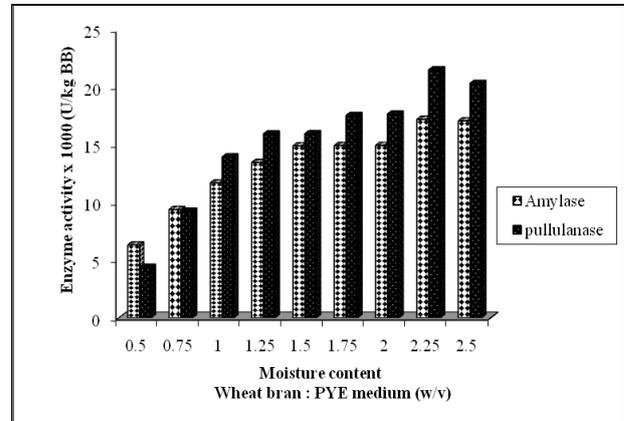


Figure 6: Effect of moisture content on amylopullulanase production by *C. thermosulfurogenes* SVM17 under solid state fermentation. The values reported in the figure are the average values of two experiments in triplicates carried out on different occasions.

The moisture content is an important factor that influences the growth and product yield in SSF (Ramesh and Lonsane, 1990). Moisture is reported to cause swelling of the substrates, thereby facilitating better utilization of the substrate by microorganisms (Kim *et al.*, 1985; Nagendra and Chandrasekaran, 1996). The data presented in the Figure 6, clearly indicates that the yield of amylopullulanase by *C. thermosulfurogenes* SVM17 increased with an increase in solid to moistening agent ratio from 1:0.5 to 1:2.5 (30 to 75%) with a maximum at 1:2.25 (73%). Any further increase in the ratio resulted in

the decrease of enzyme yields may be due to clumping of solid particles which results in the decrease of interparticle space leading to decreased diffusion of nutrients (Babu and Satyanarayana, 1996; Nagendra and Chandrasekaran, 1996; Sandhya and Lonsane, 1994; Zadrazil and Brunert, 1981). In contrast, the low moisture content leads to the decreased solubility of nutrients present in the wheat bran thereby decreases enzyme yields (Feniksova, *et al.*, 1960).

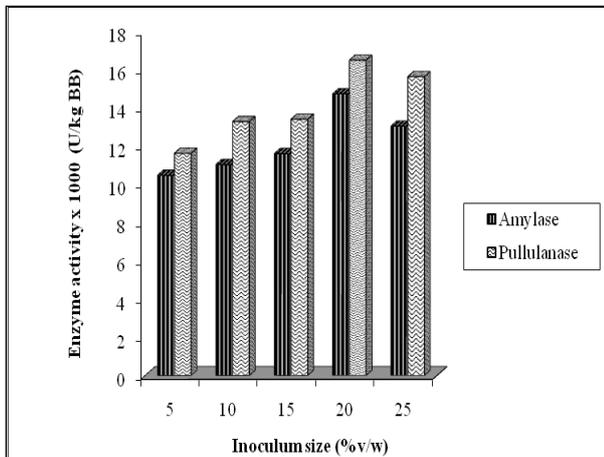


Figure 7: Effect of inoculum size on amylopullulanase production by *C. thermosulfurogenes* SVM17 under solid state fermentation. The values reported in the figure are the average values of two experiments in triplicates carried out on different occasions.

Maximum enzyme yield was obtained with 20% v/w inoculum. Further increase in inoculum concentration resulted into a gradual decrease in the enzyme yield. An inoculum size of less than 10% was found to be insufficient for growth and enzyme production (Figure 7). Similar observation was made on production of α -amylase by *B. megaterium* 16M (Ramesh and Lonsane, 1987), *B. coagulans* B49 (Babu and Satyanarayana, 1995) and *B. cereus* MTCC 1305 (Anto *et al.*, 2006). Tap water, distilled water and PYE medium used as moistening agents supported amylopullulanase production to a varied extent. PYE medium supported maximum enzyme production followed by distilled water and tap water (Table 1). A number of moistening agents such as tap water (Jaleel *et al.*, 1992; Babu and Satyanarayana, 1995; Archana and Satyanarayana, 1997), phosphate buffer (Ramesh and Lonsane, 1987), distilled water (Archana and Satyanarayana, 1997) and variety of mineral salt solutions (Babu and Satyanarayana, 1995; Archana and Satyanarayana, 1997) have been used in SSF processes for the production of enzymes.

Table 1: Effect of moistening agents on amylopullulanase production by *C. thermosulfurogenes* SVM17 under solid state fermentation

Moistening agent	Amylopullulanase production (U/kg BB)	
	Amylase activity	Pullulanase activity
Distilled water	9,221	10,086
Tap water	8,576	9,735
PYE medium	16,520	19,379

The values reported in the Table 1 are the average values of two experiments in triplicates carried out on different occasion.

The strain produced 2,600 and 1,300 U of thermostable amylase and pullulanase activities, respectively per liter in submerged fermentation. Whereas under SSF, the strain produced a maximum of 17,227 and 21,526 U of amylase and pullulanase activities respectively, per kilogram of bacterial bran. Thus the use of SSF for production of amylopullulanase by *C. thermosulfurogenes* SVM17 gave significantly higher yields as compared to those achieved in SmF and is therefore considered to be more economical.

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