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Screening of Various Raw Starches on Production of Thermostable Amylopullulanase by *Clostridium thermosulfurogenes* SVM17

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Abstract: A thermostable amylopullulanase producing *Clostridium thermosulfurogenes* SVM17 was selected for the study. Culture conditions for growth and enzyme production parameters were optimized. Enzyme synthesis occurred at temperatures between 50 and 70°C with an optimum of 60°C. The enzyme production was observed between pH 4.0 and 9.0 with an optimum of pH 7.5. Maximum growth and enzyme production was reached in 12 and 24 h of incubation, respectively. Various raw starches for amylopullulanase production were also studied. The results showed that, different raw starches influenced the enzyme yields to a varied extent. Among them, raw sago starch was found to be the best carbon source followed by wheat and potato starch, respectively on both amylase and pullulanase yields.

Key words: Amylopullulanase · Clostridium thermosulfurogenes · Raw starch degrading · Optimization · Physical parameters

INTRODUCTION

Pullulanases have industrial applications in the starch industry for the production of maltose syrups and highly pure glucose and fructose [1, 2]. Among pullulanases, amylopullulanases are interesting in starch processing industry [3] due to their specific debranching capacity in hydrolyzing either α-D-(1>6) and α-D-(1>4) glycosidic linkages [4]. Conventionally, conversion of starch to glucose and other oligosaccharides requires a two step process, namely liquefaction and saccharification before addition of α-amylase and glucoamylase.

In starch granules the molecules are densely packed in a polycrystalline state with inter and intra molecular bonds. Hence starch is insoluble in cold water and often resistant to chemicals and enzymes [5]. During liquefaction, starches are gelatinized by heating with water to improve their chemical reactivity for conversion with enzymes. These processes increases energy consumption and production cost [6]. In order to minimize the cost of gelatinization and to simplify the starch conversion process, an enzyme capable of digesting raw starch granules would provide a number of advantages. The raw starch degrading enzymes have been reported by many authors [5, 7-10]. The raw starch digesting enzymes such as α-amylase by *Bacillus* [7], *Bacillus* sp. IMD434 [5], *Bacillus* sp. IMD435 [5], *Acremonium* sp. [8], *Nesterenkonia* sp. strain F [9] and *B. amyloliquefaciens* [10], glucoamylase by *Gibberella pulicaris* [8], amylopullulanases from *Bacillus* sp. strain XAL601 [11] and *Bacillus circulans* F-2 [12] have been reported. Any improvement in the enzyme production by optimization of physical or nutritional parameters will have a direct impact on the process feasibility, performance and economics. In view of this an attempt was made to optimize culture conditions for amylopullulanase production by *Clostridium thermosulfurogenes* SVM17 and screening various raw starches for enzyme production.

MATERIALS AND METHODS

Microorganism and Culture Conditions: The bacterial strain used in the present study was selected from the isolates made from diverse sources such as compost, starch processing industrial wastes, droppings of herbivorous animals and birds in our laboratory [13]. The strain was identified as *Clostridium thermosulfurogenes*

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SVM17 by studying its morphological and biochemical characteristics as described by Hollaus and Sleytr [14], Shealth *et al*. [15]. The organism was grown anaerobically in 120 ml serum vials that contained 20 ml of pre-reduced peptone yeast extract (PYE) medium [13] composed of (g/l): NH_4Cl, 1.0; $MgCl_2.6H_2O$, 0.2; KH_2PO_4, 0.3; $Na_2HPO_4.7H_2O$, 2.0; peptone, 10.0; yeast extract, 3.0; 2.5% (w/v) $FeSO_4.7H_2O$, 0.03 ml; trace mineral solution, 10 ml; vitamin solution, 5 ml; resazurin, 0.002; 2.5% Na_2S, 20 ml; soluble starch, 5.0 and N_2 as head space gas. The medium was sterilized by autoclaving at 15 lbs for 15 min. The bottles were cooled and inoculated with 5% (v/v) exponentially grown culture and incubated at 60°C for 24 hours

Trace Mineral Solution Contained (g/l) : Nitrilo tri acetic acid, 12.8 (neutralized to pH 6.5 with KOH); $FeSO_4.7H_2O$, 0.1; $MnCl_2.4H_2O$, 0.1; $CoCl_2.6H_2O$, 0.16; $CaCl_2.2H_2O$, 0.1; $ZnCl_2$, 0.1; $CuCl_2$, 0.02; H_3BO_3, 0.01; $NaMoO_4.2H_2O$, 0.01; NaCl, 1.0; $NiSO_4.6H_2O$, 0.026; Na_2SeO_3, 0.02.

Vitamin Solution (µg/l): Biotin, 1.0; cyanocobalamine, 2.0; pyredoxinhydro-chloride, 8.0; para-aminobenzoic acid, 4.0.

Enzyme Preparation: 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 source of extracellular enzyme. The bacterial pellet was washed twice with double distilled water, suspended in distilled water and used for the estimations of cell bound enzyme activity.

Estimation of Growth: The growth was analyzed by measuring the optical density of the culture broth at absorption maximum of 660 nm using UV-visible spectrophotometer. A pinch of sodium dithionate was added to the culture broth just before taking the optical density values to remove the color development by resazurin when exposed to air.

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) soluble 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 3,5-dinitrosalicylic acid (Miller, 1959) [16]. 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.

Effect of Temperature on Growth and Enzyme Production: Effect of temperature on growth and enzyme production by *C. thermosulfurogenes* SVM17 was determined by conducting the experiments in 120 ml serum vials containing 20 ml of pre reduced PYE medium with 0.5% (w/v) soluble starch and 5% (v/v) of the exponentially growing culture inoculum and were incubated at different temperatures ranging from 40 to 80°C for 24 h. The growth and amount of enzyme produced at different incubation temperatures were estimated.

Effect of Ph on Growth and Enzyme Production: To determine the effect of pH on the growth and enzyme production, the medium was adjusted to different pH ranges from 4.0 to 9.0 using sterile 1N H_2SO_4 or 1N NaOH, prior to inoculation. A 5% (v/v) inoculum was added and incubated at 60 ± 2°C for 24 h. The growth and enzyme production in vials with different pH values were estimated.

Effect of Incubation on Growth and Enzyme Production: Growth curve experiments were conducted in 120 ml serum vials containing 20 ml of PYE medium with 0.5% (w/v) soluble starch. To this, 5% (v/v) inoculum was added and incubated at 60 ± 2°C for 72 h. Triplicate bottles were taken after every 3 h and optical density, pH, amylase and pullulanase activities were estimated.

Effect of Various Raw Starches on Enzyme Production:: To determine the effect of different raw-starches for enzyme production, *C. thermosulfurogenes* SVM17 was inoculated into 120 ml serum vials containing PYE medium supplemented with 0.5% w/v of respective raw-starches which were sterilized separately at 121°C for 1 h. The vials were incubated at 60°C for 48 h. The amount of enzyme produced was estimated under standard assay conditions described above.

RESULTS AND DISCUSSION

Optimization of Culture Conditions: An incubation temperature of 60°C was found to be optimum for the growth and enzyme production by *C. thermosulfurogenes*

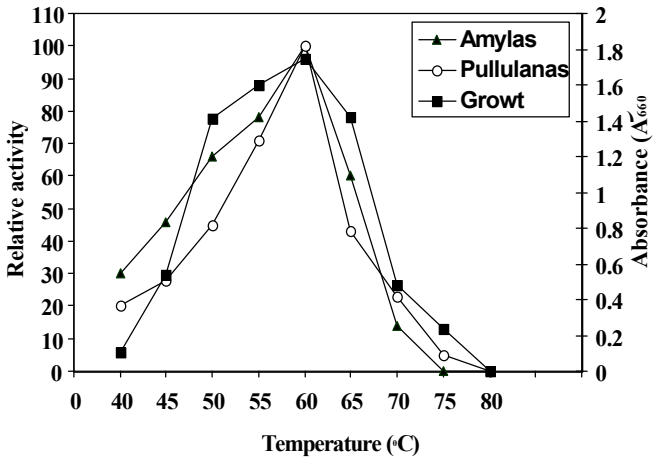

Fig. 1: Effect of temperature on growth and enzyme production by *C. thermosulfurogenes* SVM17 in submerged fermentation. Experiments were conducted in PYE medium with 0.5% starch, at different temperatures with pH 7.5 for 24h.

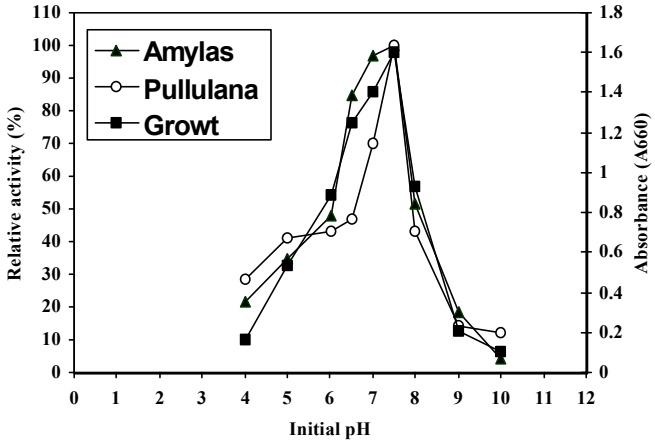

Fig. 2: Effect of initial pH on growth and enzyme production by *C. thermosulfurogenes* SVM17 in submerged fermentation. Experiments were conducted in PYE medium with 0.5% starch, at various initial pH values at 60°C for 24h.

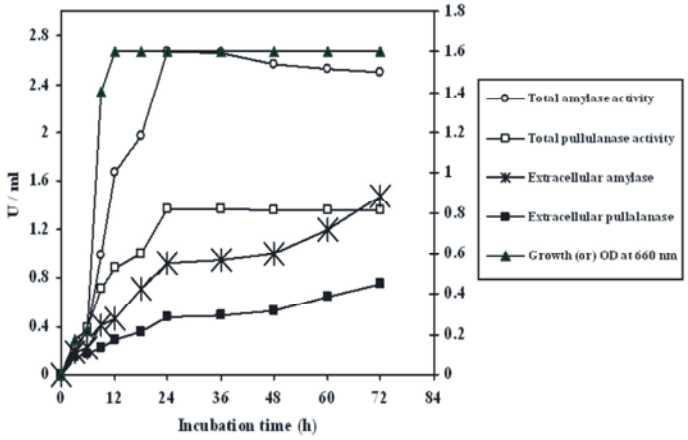

Fig. 3: Effect of incubation time on growth and enzyme production by *C. thermosulfurogenes* SVM17 in submerged fermentation. Experiments were conducted in PYE medium with 0.5% starch, at 60°C, pH 7.5 for different time periods

SVM17 (Fig. 1). At 40°C, very little growth with no enzyme secretion was observed. At 50°C, the strain showed 45 and 66 % of pullulanase and amylase activities, respectively. At higher temperature beyond 60°C, the yield of the enzyme tends to decrease gradually and at 70°C, the strain showed only 23 and 14% of pullulanase and amylase activities, respectively. At 80°C, neither growth nor enzyme production was recorded. The wide range of temperature for the growth of the strain SVM 17 was similar to *C. thermosulfurogenes* SV9 [13], *C. thermosulfurogenes* SV2 [17], *C. thermosulfurogenes* 4B [18] and *C. thermosulfurogenes* EM1 [19].

The production of enzyme was observed between pH 4.0 and 9.0. The optimum pH for growth and enzyme production was 7.5. At pH 4.0, little growth and low amount of enzyme having 21.64 and 28.54% of amylase and pullulanase activities, respectively were observed. At pH 9.0, very negligible amount of enzyme was recorded (Fig. 2). The strain SVM 17 had a broad pH range for growth and enzyme production similar to *C. thermosulfurogenes* SV9 [20], *C. thermosulfurogenes* SV2 [17], *C. thermosulfurogenes* 4B [18] and *C. thermosulfurogenes* EM1 [19].

Figure 3 shows the pattern of growth and production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM17. Maximum growth and enzyme production was reached in 12 and 24 h of incubation, respectively and further incubation upto 72 h did not show much influence on growth and enzyme production. However, a fall in pH of the medium from 7.5 to 4.8 was observed due to production of acetic acid and lactic acid. With the increase in incubation time (72 h), the extra cellular pullulanase production has increased from 145 (at 3 h) to 756 U/l (at 72 h) and the extra cellular amylase from 183 (at 3 h) to 1471 U/l (at 72 h) with concomitant decrease in respective cell bound enzymes. This may be due to the loss of binding capacity of the enzyme with the cells or lysis of the cells occurring in the culture broth. However, Madi *et al.* [21] observed with *C. thermosulfurogenes* EM1 have reported that on prolonged incubation time also these enzymes remained as cell bound (more than 95% for 60 h) with starch or maltose as substrate. Similar observations are made with *C. thermohydrosulfuricum* [1] and *Thermoanaerobacter* B6A [22].

The enzyme production was found to be optimum at growth temperature of 60°C, initial pH of 7.5 and incubation period of 24 h. Under these optimum conditions, the enzyme showed 2,600 and 1,300 U of thermostable amylase and pullulanase activities, respectively per litre of culture broth using PYE medium containing 0.5 % soluble starch as carbon source. The yields of amylolytic enzymes producing maltose and maltooligosacchaides in submerged fermentation vary in different organisms. *C. thermohydrosulfuricum* 39E [1], *Clostridium* sp. strain EM1 [23], *C. thermosulfurogenes* SV2 [24] and *Thermococcus hydrothermalis* [25] produced about 180, 250, 910 and 30U of thermostable pullulanase, respectively per litre of culture broth. Swamy and Seenayya [13] reported that *C. thermosulfurogenes* SV9 produced about 400 and 640 U of thermostable α-amylase and pullulanase, respectively per litre of culture broth. In another study, the strain *C. thermohydrosulfuricum* Sol 1 produced about 1,100 and 2,500 U of amylolytic and pullulytic activities, respectively per litre of culture broth. Whereas, *C. thermohydrosulfuricum* Uel 1 showed 2,700 and 5,500 U of amylolytic and pullulytic, activities, respectively per litre of culture broth [26]. In continuous cultures, *C. thermosaccharolyticum* and *Thermoanaerobacter acetoethylicus* produced 5,680 and 5,670 U of amylopullulanase, respectively per litre of culture broth, respectively [27]. It is clear from the above reported work that the enzyme yields of the strain SVM17 were higher when compared to the yields of *C. thermosulfurogenes* 39E, *Clostridium* sp. strain EM1, *C. thermosulfurogenes* SV2, *C. thermosulfurogenes* SV9 and *Thermococcus hydrothermalis* and the yields were lower than that of *C. thermosaccharolyticum* Ue11 and Sol1.

Influence of Raw Starches on Production of Thermostable Amylopullulanase: The results presented in Table 1 shows that different starches influenced the enzyme production to a varied extent. Raw sago starch was found to be the best carbon source followed by wheat and potato starch, respectively on both amylase and pullulanase production. Relatively, very low amylase and pullulanase activities were observed when raw starches of corn, soluble starch (uncooked), tapioca, barley, bajra and rice were used as carbon sources. Similar findings was reported by Marlida *et al.* [8] where a high amylase activity was produced from *Acremonium* sp. using raw sago starch, followed by raw rice and tapioca. The alkaline thermostable amylopullulanase from *Bacillus* sp. strain XAL601 [11] and amylase-pullulanase enzyme from *Bacillus circulans* F-2 are only the enzymes that can digest raw starch granules effectively [12]. The amylase I and amylase II of *Bacillus* sp. WN11 were capable of digesting raw corn and potato starch [7]. The α-amylase from *Bacillus* sp. IMD435 hydrolyzed raw corn and raw

Table 1: Effect of various raw starches on production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM17 in SmF

	Amylopullulanase					
	Amylase activity (U/L)			Pullulanase activity (U/L)		
Raw starch	EC♦	CB*	Total	EC♦	CB*	Total
Sago starch	478	932	1410	248	784	1032
Wheat starch	296	825	1120	98	779	878
Corn starch	98	189	290	77	140	217
Soluble starch	47	297	344	70	169	238
Tapioca starch	110	88	199	80	61	140
Barley starch	123	182	305	77	69	146
Bajra starch	227	95	321	172	64	234
Potato starch	225	273	497	102	180	282
Rice starch	130	108	238	69	60	130

Extracellular, * Cell - bound Experiments were conducted in 120 ml serum vials containing PYE medium at 60°C for 24 h with 0.5 % (w/v) concentration of the respective raw starch. Cells were transferred three times in the medium containing the corresponding raw starch. The enzyme activities were assayed under standard assay conditions.

rice starch. Potato starch was also hydrolyzed, but to a lesser extent [5]. The α-amylase from *Bacillus* sp. YX-1 hydrolyzed raw corn, wheat and potato starch granules [28]. The α-amylase from halophilic *Nesterenkonia* sp. strain F hydrolyzed raw wheat and corn starch [9]. Immobilized α-amylase from *B. amyloliquefaciens* hydrolyzed raw potato starch [10]. β-amylase from *Emiricella nidulans* [29] hydrolyze barley and wheat more effectively. The enzyme from strain T-20 exhibited highest activity with raw corn starch and significant activities were observed with raw potato and cassava starches [30]. The production of amylases on raw starches may depend on the properties of different carbon sources and the capability of the microorganisms to utilize them.

CONCLUSION

From the results it is evident that the optimum temperature, pH and incubation time for growth and enzyme production were found to be 60°C, 7.5 and 24 h, respectively. Raw sago starch found best for production of enzyme among different raw starches screened. The ability to hydrolyze raw starch such as sago starch (which is abundantly available) would help overcome the gelatinization step during liquefaction there by minimizing the cost of starch processing.

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