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Production of Thermostable α-Amylase and Cellulase from *Cellulomonas* sp.

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Abstract A bacterium, isolated from rabbit's waste and identified as *Cellulomonas* sp., had cellulase and thermostable α-amylase activity when grown on wheat bran. Maximum activity of thermostable α-amylase was obtained by adding 3% soluble starch. However, soybean oil (1 ml l^{-1}) could increase the production of α-amylase and cellulase in wheat bran. The α-amylase was characterized by making a demonstration of optimum activity at 90°C and pH 6–9, with soluble starch as a substrate. The effect of ions on the activity and the stability of this enzyme were investigated. This strain secreted carboxymethyl cellulase (CMCase), cellobiase (β-glucosidase), and filter paperase (Fpase) during growth on wheat bran. Carboxymethylcellulase, cellobiase, and Fpase activities had pH optima of 6, 5.5, and 6, respectively. CMCase and cellobiase activities both had an optimum temperature of 50°C, whereas Fpase had an optimum temperature of 45°C.

Key words: *Cellulomonas* sp., thermostable α-amylase, cellulase

Amylases are widely used in textile, confectionary, paper, brewing and alcohol industries [11]. They are utilized where rapid hydrolysis of starch is required or the viscosity of starch slurry needs to be lowered. α-Amylase is produced by a wide range of microorganisms, and the thermophiles are no exception. The enzyme hydrolyses α-1,4 glucosidic linkages in amylose, amylopectin, and glycogen in an endo-cleavage fashion, the end products of its action being sugar. Bachina *et al.* [2] reported the investigation of 237 strains that resulted in thermophilic *Bacillus*, *Actinomycetes* fungi for the production of α-amylase. However, there is no report made about production of thermostable α-amylase from *Cellulomonas* sp.

The biodegradation and bioconversion of lignocelluloses into useful products and biological alleviation of pollution from lignocelluloses wastes are enormous environmental challenges. Lignocelluloses gained much attention during the 1980s as a possible source of single-cell protein (SCP) to satisfy and increase worldwide demand for new protein foods and enzyme production [5]. The widespread application of enzymes in feeds to monogastric animals is a recent development. Many enzymes like amylases, glucoamylases, glucanases, cellulases, pentosanases, proteinases, and xylanases are used in animal feed [8]. Delignification of lignocelluloses material by white rot fungi is of great interest and has been investigated not only to improve the digestibility of wood or straw for animal feed but to reduce costs for pulp and paper industry as well [10, 13].

In this report, we study the biodegradation of wheat bran and the production of cellulase and thermostable α-amylase during the bioprocessing of wheat bran.

Materials and Methods

For the enzyme production, the microorganisms isolated from rabbit waste were grown in the following medium (gl^{-1}): wheat bran 10.0; peptone 0.5; KH_2PO_4, 2.0; $(NH_4)_2SO_4$ 1.4; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2$, 0.3; $FeSO_4 \cdot 7H_2O$, 0.005. pH was adjusted to 7.0. The medium receives a 10.0% (v/v) inoculum of a pre-grown culture and is incubated at 28°C on an orbital shaker.

Enzymes Assay

Cellulase activity in the culture supernatant fluid was assayed by the filter paper test. Filter paper was hydrolyzed by the enzymes for 1 h at 45°C and pH 6. The reducing sugar produced was measured (as glucose) using dinitrosalicylate as described by Mandel and Weber [10]. Cellobiase activity was assayed according to the method of Okada [12]. Protein concentration was measured according to the method of Bradford [3] using bovine serum albumin as a standard.

Endoglucanase was determined by its activity against carboxymethylcellulose (CMC). This was assayed by mixing

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1 ml of an appropriate enzyme dilution with 4 ml of CMC (10 gl^{-1}) in 25 mM potassium phosphate (pH 6), and the mixture was incubated at 50°C. After 15 min, the reaction was stopped by adding 2 ml of dinitrosalicylate. The resulting mixture was boiled for 10 min and a reducing sugar content was measured by absorbance level at 640 nm [14].

Amylase was estimated from reducing sugar liberation in 2 ml reaction mixtures containing 2% soluble starch, 0.1 M phosphate buffer, pH 6.0, and 0.1 ml of cell-free culture fluid and incubated for 10 min at 65°C.

Enzyme Characterization

Studies conducted on optimal pH and temperature and stability levels of extracellular and endocellular enzymes of *Cellulomonas* isolates were carried out.

RESULTS AND DISCUSSION

The isolated microorganism was a Gram-positive, oxidase positive, facultative anaerobe, nonspore-forming bacteria and it could use cellulose as the only source of carbon and energy. It was identified as *Cellulomonas* sp. according to biochemical tests (Table 1).

Amylase Production by *Cellulomonas*

This strain produced thermostable α-amylase. Maximum activity of the enzyme was recorded at 3% soluble starch (Fig. 1). These enzymes showed a continuous rise in its activity up to 3% starch concentration. At 5% concentration, a slight decrease in the enzyme activity was observed. The increase in the enzyme activity by increasing the substrate concentration followed the law of mass action. The activity was low at lower concentration because most of the active sites of the enzyme molecules remained without any substrate. As the substrate concentration increases, a larger number of substrate molecules will bind to the active sites of the enzyme, and the number of microorganisms will increase. During growth on starch as the sole carbon sources in mineral salts medium, the extracellular amylase levels in culture fluids reached a maximal level of about 100 units (μmol/ml; one unit defind as μmol sugar per 1 ml reaction mixture after making an addition of 0.1% maltose to preculture.

Table 1. Biochemical and morphological properties of *Cellulomonas* sp.

Test	Reaction	Test	Reaction
Acid fast	-	Rafinose	-
Reduction of nitrate	+	Lactate	-
Catalase	+	Cellulose	+
Yellow pigment	+	Acid from dextrin	+
Motility	-		

(+) positive and (-) negative.

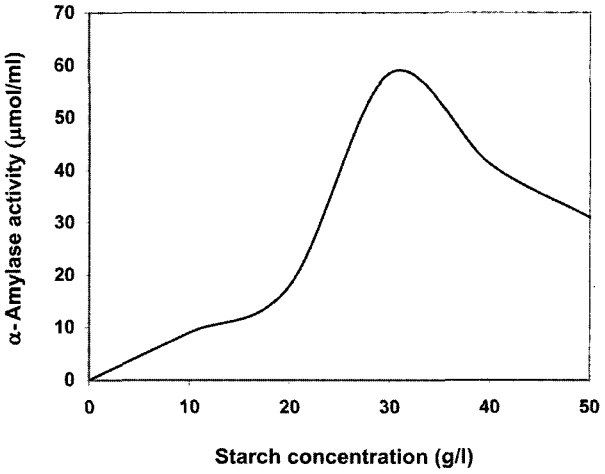

Fig. 1. The effect of substrate concentration on α-amylase activity.

During the growth of the *Cellulomonas* on wheat bran, extracellular α-amylase level rose initially and stayed constant with CMCase and FPase activity after 3 days of growth at 27°C (Table 2). Therefore, the presence of cellulose and cellulase (CMCase and FPase) did not cause the inactivation of amylase in a culture fluid. However, Busch and Stutzenberger [4] showed that when *Thermomonospora* were grown on a mixture of cellulose and starch, α-amylase was repressed via inhibition of maltose uptake by cellobiase.

Effect of temperature: The effect of temperature ranging from 30°- 100°C on the activity of amylase was evaluated (Fig. 2). The enzyme of *Cellulomonas* exhibited an increase in its activity from 60°- 100°C. When a longer time period was taken for boiling of α-amylase, the activity of this unusual α-amylase did not change.

Effect of pH: The data on the effect of different pH of buffer on the activity of α-amylase of *Cellulomonas* are given in Fig. 3. α-Amylase showed a maximum activity at

Table 2. Production of cellulolytic enzymes and α-amylase by *Cellulomonas* in liquid media.

Liquid media	Endoglucanase (Uml^{-1})	Exoglucanase (Uml^{-1})	α-Amylase (Uml^{-1})
Wheat bran+T80 (1%)	1.5	2	ND
Wheat bran+biotin	1.5	1.75	ND
Wheat bran+Soya bean oil	2	3	80
Potato dextrose	0	0	15
Wheat bran	3	3	30
Corn starch	0	0	20
Soluble starch	0	0	60

Process time and pH were 3 days and 7, respectively.

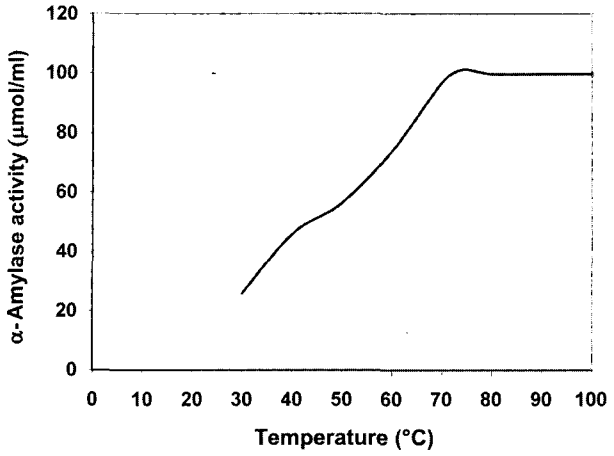

Fig. 2. Effect of temperature on the activity and stability of α-amylase.

pH 6–9. Production of thermoacidophilic thermoalkaline and alkaline α-amylase has been reported by Bajpai *et al.* [1] and Kim *et al.* [9]. Also in our work, the α-amylase from *Cellulomonas* was highly thermostable. Table 3 shows the activities of the amylase assayed in the presence of different metal ions. The enzyme was inhibited in the presence of Hg^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, and Pb^{2+}. Partial inhibition of activity was observed in the presence of Zn^{2+}. Igarashi *et al.* [7] purified 53 kd amylase from an alkaliphilic *Bacillus* with high yield (3%), and inhibition of this enzyme by Zn^{2+} and Hg^{2+} ions was reported by them as well.

Production of Cellulolytic Enzymes and α-Amylase by *Cellulomonas* sp.

The enzyme production was investigated and different liquid media were chosen. The results are shown in Table 2.

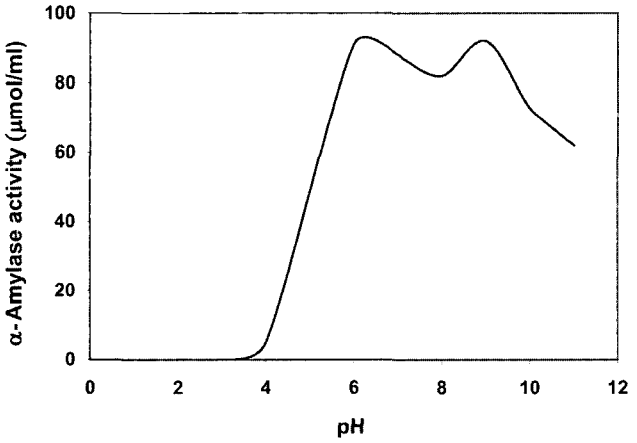

Fig. 3. Effect of pH on the activity and stability of purified α-amylase from *Cellulomonas* sp.

Table 3. Effect of different metal ions on activity of amylases from *Cellulomonas*.

Metal ion	% Activity
NaCl	100
KCl	100
$CaCl_2$	100
$CuSO_4$	98
$ZnSO_4$	9
$Pb(CH_3COO^-)_2$	8
$FeCl_3$	0
$FeSO_4$	14
$ZnCl_2$	85
$HgCl_2$	0

The activity of enzyme was assayed in the presence of the different salts at a final concentration of 1 mM for 30 min.

As shown, wheat bran in a liquid media is actually the best media for the production of enzymes. Tween 80 and biotin did not increase the enzyme production of *Cellulomonas* sp. Tween 80 can increase the production of cellulase in fungi. However, Soya increased the production of extracellular α-amylase of this microorganism, and Soya oil meal was induced for making a production of α-amylase by *Bacillus*.

Multi-enzymes activities were measured in the supernatant after removing the microorganisms and wheat bran by centrifugation. As shown in Table 4, the isolated microorganism secreted both endo- and exo-glucanases, with α-amylase in the culture medium.

Determination of pH and Optimal Temperature and Cellulase Stability

To determine the optimal temperature for enzyme activities, assays were performed at 20°–90°C, pH 6.0, except for cellobiase assays, which were conducted at pH 5.5. Assays for optimal pH were performed at 50°C or 45°C for FPase in the pH range 2.5 to 9.0. Cellulase activity was determined with supernatants which were culture grown for 3 days on a whatman No. 1 filter paper. The optimal pH and temperature of the cellulase were pH 6.0 and 45°C, pH 6.0 and 50°C, and pH 5.5 and 50°C for FPase, CMCase, and cellobiase, respectively.

Table 4. Activities of multi-enzymes from *Cellulomonas* at 80°C in 10 min.

Enzymes	A (Uml^{-1})	B (Uml^{-1})
Exoglucanase	2.5	2
Endoglucanase	3.5	1.5
α-Amylase	30	23

A=before thermal treatment.
B=after thermal treatment.

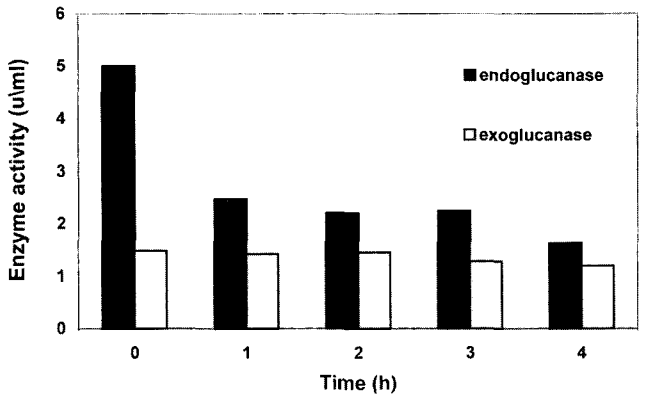

Fig. 4. Thermal stability of endoglucanase and exoglucanase in *Cellulomonas* sp. at 60°C.

Enzyme stability was investigated by incubating the supernatants of the strain at 20-90°C for 10 min, without the substrate. Relatively short incubation periods at 60°C resulted in substantial losses in enzyme activities and at the end of a 4 h incubation period, exoglucanase and CMCase activities were reduced by 20% and 70%, respectively (Fig. 4). CMCase was more sensitive to the heat treatment and its activity was reduced by 60% at 70°C for 10 min (Fig. 5). Thermal treatment on activities of multi-enzyme is shown in Table 4. It was obvious that endo- and exoglucanases of *Cellulomonas* sp. exhibited high stabilities at 40°C. However at 60°C, endoglucanase is reduced more than exoglucanase.

In summary, *Cellulomonas* sp. isolated from rabbit waste grew on wheat bran and produced thermostable α-amylase which is used in many industrial technologies. Although Busch and Stutzenbrger [4] showed inactivation of α-amylase in *Thermomonaspora* species during growth on cellobiase, the isolated microorganisms had α-amylase and β-glucosidase activity during growth on wheat bran with production of 80% protein. Amylase and cellulase are food additives of poultry [6] and this isolate could be used in industry.

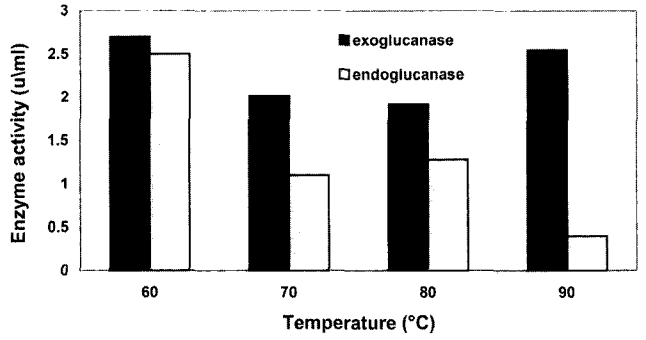

Fig. 5. Optimal temperature for exoglucanase and endoglucanase activity of *Cellulomonas* sp. thermally treated for 10 min.

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